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DEVELOPMENT OF IN VITRO PROTOCOLS TO ENHANCE SECONDARY METABOLITE PRODUCTION FROM TURMERIC (*CURCUMA LONGA* L.)

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Plant and Environmental Sciences

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by
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Accepted by:
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James Rieck

ABSTRACT

Plant and organ culture can readily be applied to the field of medicinal plant and secondary metabolite production. Many studies that have been conducted attest to the utility of in vitro methods for preservation of genetic diversity, propagation of clonal individuals for use in commercial field planting, and production of metabolites directly from culture. Experiments have also shown that tissue culture is an important tool for enhancement of medicinal compound production utilizing various methods including elicitation. This thesis focuses on production of metabolites using an in vitro system. An overview in the form of a literature review is provided, which details the many ways that tissue culture has been applied to medicinal plants. Two major concerns are addressed experimentally, quantity of biomass and concentration of compounds in that biomass. Quantity of biomass was evaluated in a time course factorial experiment employing three different concentrations each of methyl jasmonate (MeJa) and benzyladenine (BA). MeJa negatively impacted biomass accumulation while BA had a positive impact on biomass accumulation. Sugar use was found to directly correlate with dry weight production, and a system for long-term growth of plant materials was developed. Concentrations of compounds in the biomass were explored by observing effects of media additions of proline, a natural source of proline (Gropro), MeJa, phenylalanine, chitosan, and nitrogen stress. MeJa and phenylalanine were utilized in a factorial experiment, and both compounds reduced biomass and secondary metabolite production. Proline, Gropro, MeJa, chitosan, and nitrogen stress were evaluated in comparison with a control in a separate experiment. Only one treatment, nitrogen stress, was successful in upregulating

metabolite production. Nitrogen stress was found to increase total phenolic production. It was also successful in causing a nearly significant ($p = 0.1207$) increase in antioxidant activity from extracts. Sugar and nutrient status are critical factors relating to our main goals, enhancement of biomass production and enhancement of chemical concentration. As a result of these two bodies of work, we conclude that future experiments should evaluate nutrient stressed plants in elicitation studies where sugar is optimized for maximum biomass production. Other upregulation methods would be employed in this new system to further enhance chemical production.

DEDICATION

This thesis is dedicated to my mother, Lynn Cousins, who gave me more than I can ever repay and taught me more than anyone else ever can; to my father, Mike Cousins, who has been an ever present example of the person I should forever strive to be; to my grandfather, Warren Cousins, for his many lessons and never-ending strength of character; and to my best friend, Emily Gaunt, who has shown me what really matters in life.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

The uses of medicinal plants vary with culture, but these valuable resources for production of medicinal products have been accepted and utilized in some way by almost every culture throughout history. Modern synthetic drugs and allopathic medicine have been preferred in the US and many other developed countries for much of the 20th century. The emergence of modern environmentalism and back-to-nature movements has brought about a renaissance in large scale use of medicines derived from plants. Thorough characterization of natural products is a prerequisite to modern exploration and validation of herbal treatments. In 1999, the worldwide market for medicinal plant products surpassed $15 billion with some of the top markets being Europe, $2.4 billion; Japan, $2.7 billion; mainland Asia, $2.7 billion; and the United States, $3 billion (Glasser, 1999). By 2003, estimates for the global medicinal plant market had exceeded $60 billion (World Health Organization, 2003). The World Health Organization has projected that global demand for medicinal plants could reach $5 trillion by the year 2050 (Sharma, 2004). As of 1991, approximately 120 prescriptions worldwide were plant based derivatives with US medicine drawing roughly 25% of its pharmaceuticals from plant produced medicinal compounds (Payne et al., 1991). This percentage has remained relatively constant over the years, with similar percentages being found in the studies of Farnsworth and Morris (1976), Farnsworth and Soejarto (1985), and Grifo et al. (1997). Unfortunately, there are major quality control issues with medicinal plants and their

production, and roughly 60% of commercial medicinal products sold in the US would fail to pass tests for quality, consistency, and potency (Glaser, 1999). Marcus and Grollman (2002) found problems with medicinal plant products including lack of standardization, contamination, and lack of proper reporting of adverse effects; they concluded that new regulations are needed.

Currently, only 10% of medicinal plant species are cultivated, and the remaining 90% are harvested from wild populations (Julsing et al., 2007). Excessive harvesting can diminish native populations and erode genetic diversity while skewing the survivors toward accelerated development and reproduction. Domestication and acceptance of good agricultural practices are necessary steps toward standardization and commercial production. In traditional medicinal crops, processes of domestication required hundreds of years to harness the medicinal compounds that plants produce for their own defensive or protective purposes. Prior to the advent of modern analytical protocols and cultivar development techniques, biochemical products of medicinal plants were enhanced and developed via intentional and unintentional cultivar improvement. In a similar fashion, deliberate and inadvertent breeding and selection by more primitive cultures from ancient times did much to produce the agricultural products we utilize today.

Biotechnology has a role to play in accelerated development of reliable plant medicines. The recent focus on genetic transformation may concern some modern consumers for religious, cultural, or environmental reasons. Plant tissue culture is an example of a biotechnology with a multitude of applications to medicinal plant improvement programs, and it is not included in the controversy involving genetically

modified organisms. As a result, tissue culture will serve as an indispensable tool for the identification of useful medicinal plant varieties and for the alleviation of strain often placed on native populations.

Plant tissue culture has been a preferred biotechnology for applications regarding medicinal plants for many years. Originally, the envisioned growth and synthesis model involved using systems similar to those used in microbial fermentation to grow large volumes of undifferentiated callus cells suspended in liquid medium. Even after 30 years of active research, there are surprisingly few commercial applications that utilize this technology. According to Alferman et al. (2003), many attempts have been made to use plant cell culture for commercial production of plant secondary metabolites; however, most of these attempts have not been feasible. Only four commercially viable in vitro secondary metabolite production systems have been created including shikonin (*Lithospermum erythrorhizon*), ginsenosides (*Panax ginseng*), purpurin (*Rubia akane*), and paclitaxel (*Taxus* spec.). Alferman et al. (2003) resolved that the lack of significant application was due to several factors including a lack of storage cells for accumulation of secondary metabolites. In addition, Preil (2005), blamed lack of progress specifically on the high cost of bioreactors, slow growth of plants, and low yields of active metabolites.

The over-expression of differentiated roots following transformation with *Agrobacterium rhizogenes* has created the subfield of hairy root culture. Hairy root cultures often have greater synthetic capacity than undifferentiated cell suspension cultures. As a result, many researchers have found great potential for direct production of

medicinal biomass using such systems. Hairy root culture will be further discussed later in this review.

Whole plant and organ culture systems are more difficult to commercialize in bioreactors than cell and hairy root culture. Thus, these systems have received the least attention with regard to application of tissue culture to production of medicinal plants. This review will focus on whole plant and organ culture for preservation of plant material, production of young starter plants, production of biomass that contains active secondary chemistry, and enhancement of secondary metabolite production. Turmeric (*Curcuma longa* L.) will be referenced as a model system for the different methods discussed. Since it is little known in American horticulture, a thorough background of this plant will be provided as part of this discussion.

Discussion

I. A Tool for Preservation

Collecting germplasm from distant sites around the globe with the intent of returning years later to find that same clone is often impossible because one's ability to do so depends on the plant's life cycle, urbanization, societal developments, and regional politics. Breeders and biologists need stable means for storage of plant material for reference and use. Medicinal plants display significant intraspecific variation with regard to many complex traits (including the phytochemical profiles). Seedling progeny of outcrossed heterozygous individuals (likely in most medicinal species) will vary with regard to the amounts and ratios of metabolites they produce. Creating clonally

multiplied lines of wild-collected plants during biochemical characterization may be particularly prudent if the plant exhibits sterility, low seed production, slow growth, or long generation time. The use of tissue cultured plantlets for storage of genetically distinct individuals has the added benefit of minimizing the risk of inadvertent transfer of pests during transcontinental transport of living plant material.

Asexually propagated crops maintained by cuttings and crown divisions are particularly difficult to maintain as germplasm since propagation often spreads pathogens. Many medicinal plants that are also used as culinary herbs have been in national germplasm collections for many years. Tissue culture systems have been developed to facilitate maintenance and dissemination of materials from these national plant germplasm collections. Subculture cycle time can be increased by storage at lowered temperature or extended indefinitely where cryopreservation is employed. The feasibility of cryopreservation has been established for both herbaceous (e.g. celery, chamomile, ginseng, hops, horseradish, and wasabi) and woody (e.g. citrus, eucalyptus, poplar, neem, rose, and tea) species (Towill and Bajaj, 2002). There has been limited implementation of cryopreservation protocols in germplasm banks due to disunity with regard to the many groups involved in these projects (government entities, non-profit foundations, and breeders); this lack of unity has resulted in an inability to provide long-term funding to offset the costs of protocol development and infrastructure while maintaining security (Reed, 2002).

II. Micropropagation and Starter Plants for the Field and Greenhouse

Tissue culture of medicinal plants also displays great utility in both field production processes and controlled environment protocols for use in laboratories, growth chambers, and greenhouses. Micropropagation techniques allow for the rapid creation of millions of genetically identical plants – many more than could be produced through conventional propagation methods. This can be critical when the medicinal crop is slow growing and has a limited geographic range. Many medicinal species have been collected to near extinction. In vitro methods have been used on *Dendrobium* orchid species in China to stave off pressures from over-collection and habitat destruction (Vanisree and Tsay, 2007). *Hydrastis canadensis*, goldenseal, is an endangered medicinal plant for which tissue culture has been used to conserve germplasm and prevent damage to native populations (He et al., 2007). Tissue culture methods have been developed for and applied to many other endangered medicinal plants including: *Curculigo orchioides* (Suri et al., 1999), *Podophyllum hexandrum* (Nadeem et al., 2000), *Hypoxis hemerocallidea* (Ndong et al., 2006), and *Saussurea involucrate* (Guo et al., 2007).

An early step in maximizing biochemical production from a particular species is the selection of "elite" individuals with exceptionally high metabolite production potential. Tissue cultures can serve as quality stock plants for greenhouse, field, or wild-simulated production systems. In vitro systems are capable of quickly supplying large numbers of genetically uniform individuals. Thus, these systems are critical to meet the plant material demands of high-turnover, continuous-cropping schemes for field or greenhouse production of medicinal plants. Both fixed facility costs and operational

laboratory costs of plant production are higher for in vitro culture labs than they are for field nurseries. Sterile material rarely has a high enough value to warrant these additional expenses.

In vitro systems supply sterile material that is free from outside contamination. As a result, these systems are particularly useful for production of starter plants. This tissue can be kept free of bacterial or viral infections or infestations as a result of the inherent sterility and high quality control involved in propagation. As a result, the plant material produced for use in planting operations is of higher quality than that which would be derived from typical vegetative propagation. Higher quality planting material minimizes losses in productivity normally incurred due to diseases spread in propagation. Such systems also alleviate problems resulting from disease and pest entrance into production facilities along with propagules. Since environmental characteristics have been shown to impact medicinal chemical production by plants, the use of controlled environments has been employed for manipulation of humidity, temperature, day length, and carbon dioxide concentration (Fonseca et al., 2006).

In vitro material with fully formed root and shoot systems may be produced that is completely prepared for autotrophic existence outside of the culture vessel. This accelerates production beyond rates possible with slower operations that are preformed through the use of seed planting because tissue culture alleviates the need for seed germination and growth periods. In aeroponic or hydroponic systems such as those described by Hayden (2006) and Stutte (2006), in vitro plant materials were capable of

rapid acclimatization during transfer of well-developed material from culture vessels to soilless systems for growth.

The in vitro derived material can be treated or challenged prior to its insertion into a less rigorously controlled, natural light production environment such as a field or greenhouse. This treatment is designed to enhance metabolite production by the plant following transfer to a less costly growth environment.

III. Bioreactors and Biomass

Bioreactors are vessel systems that allow monitoring and modification of the organism without violating asepsis. Plant tissue culture is conventionally a batch process with inoculum and all nutrients being supplied in a semi-solid gel on the day of initiation. Bioreactors are liquid systems where oxygen, water, nutrients, and growth regulators may be added periodically (fed-batch) or continuously over the duration of the culture period. Greater plant mass and more control of metabolic processes justify the progression of technology from batch to fed-batch or continual feed systems.

Plant tissue culture has been explored as a method to produce secondary metabolites for many years. Recently, systems for active chemistry and biomass production that might some day be applicable on the commercial scale have been developed (Zobayed et al., 2004a; Yan et al, 2005). Chemical syntheses of organic molecules with multiple chiral centers and complex structures are difficult and inefficient. Plant tissue culture systems are a valuable asset because of their potential to

replace complex and inefficient syntheses with natural processes that likely have minimal environmental impact.

Before a direct discussion of in vitro production of medicinal plants through plant and organ culture, the historic knowledge gained in cell culture will be discussed relative to our current understanding of medicinal plant biology. The first experiments conducted used callus and suspension culture of undifferentiated cells as a method for production of secondary metabolites. Despite significant limitations, cell suspension culture has been successfully exploited commercially for production of secondary metabolites. Taxol®, paclitaxel, is a compound with anticancer properties that was originally extracted from leaves and bark of plants of the genus *Taxus.* This compound is being successfully produced via cell suspension culture by Phyton Biotech (Germany) in 70,000 L bioreactors (Wink et al., 2005). In most instances, however, callus culture fails to produce significant quantities of medicinal compounds because of the need for compartmentalization of cellular synthetic processes which require differentiation for proper pathway functionality (De Luca and St Pierre, 2000). Immobilization of suspension cultures was originally conceived as a possible enhancement of the system to allow the productive cells to be maintained while the bioactive compounds were removed with the majority of the media (Baldi et al., 2007). Immobilization of *Capsicum* cell cultures allows for continual production of capsaicin in vitro (Johnson et al., 1990).

Many successes have been achieved in medicinal plant production using hairy root culture. Hairy roots are created through the transformation of normal roots using *Agrobacterium rhizogens* or *Agrobacterium tumefaciens*. Many of the aspects of hairy

root culture have seen great improvement in recent history with advances in secondary chemistry production, pathway engineering, induction of metabolite excretion, and elicitation; more advancements are expected in the near future (Guillon et al., 2006a; Guillon et al., 2006b). Fons et al. (1999) found that normal roots and hairy roots of *Plantago lanceolata* produced similar amounts of major phenolic constituents though hairy roots accumulated more biomass. Hairy root cultures of *Saussurea involucrata* produced larger quantities of some major metabolites than wild plants, and media type influenced phenylpropanoid accumulation (Fu et al., 2006). Yan et al. (2005) developed a hairy root system for *Salvia miltiorrhiza* for secondary metabolite production that employed repeated media addition for prolonged biomass accumulation, elicitor for metabolism induction, and resin to collect metabolite. The system functioned successfully though there was admittedly a need for further optimization. Hairy root cultures of *Rauvolfia micrantha* were capable of indole alkaloid synthesis (Sudha et al., 2003). These compounds normally reach maximal levels in this species after four years of age and are affected by climate. As a result, such a system for hairy root production would be advantageous for this and other plant metabolites with similar production constraints.

An alternative to cell culture and hairy root culture and a focus of this review, plant and organ culture allow for the differentiation and compartmentalization of metabolic processes driving anabolism of secondary metabolites. Shoot cultures have traditionally been applied to uses for micropropagation in medicinal plant culture. There are, however, many metabolites that can be produced directly in shoot cultures of plant materials (Kreis, 2007). For example, *Artemesia annua* did not produce artemesin in

callus or hairy-root cultures. Shoot cultures did produce artmesin, and the highest levels were obtained when the shoots developed as plantlets with roots in vitro (Ferreira and Janick, 1996). In vitro shoots and roots of *Centaurium pulchellum* were found to contain secoiridoids and xanthones with decreases in some metabolites and increases in others when in vitro tissue was compared with tissue produced in natural growing plants (Krstic, 2003). In order for production of podophyllotoxin by *Linum flavum* L. to be improved, differentiation was required (Van Uden et al., 1991).

Large bioreactor size, tissue sensitivity to mechanical shear forces, and low growth rates make adaptation of microbiological immersion type mechanisms unsuitable for use as bioreactors for whole plant or organ culture. Temporary or partial immersion is viewed as an important distinction to allow plant and organ cultures to be oxygenated with less mechanical shear than would be realized in many stirred bioreactors. To our current understanding, the largest liquid bioreactor laboratory for plant production is Bioplantas Ltd. (in Cuba). This company utilizes a scaled-up temporary immersion system for production of banana, pineapple, and coffee plants. When compared to agar based culture systems, liquid cultures yield plantlets with higher dry mass that are capable of more photosynthesis and growth upon transfer to soil while having a lowered cost of production (Gonzales, 2005). Growth was found to be slow immediately following transplantation into ex vitro conditions with in vitro derived leaves functioning both as storage organs and as autotrophic organs with limited photosynthetic capability (Van Huylenbroeck et al., 1998), but higher photosynthetic rates were observed in leaves formed after transplant (Reuther, 1988). Zobayed et al. (2004b) found that plants grown

in temporary immersion systems had more dry weight and fresh weight than plants grown in other systems. Aragon et al. (2005) concluded that higher rates of photosynthesis and enzyme expression were seen during acclimatization of plants grown in temporary immersion culture. There are three main reasons for use of temporary immersion culture: supply and transfer of nutrient to the plant while maintaining proper oxygenation, significantly reduced hydrodynamic and mechanical shear force, and lowered cost requirement (Liu et al., 2004). The temporary immersion system used in our laboratory constitutes a simplified system for attaining temporary immersion that does not require costs associated with mechanical linkages between pumps and vessels (Adelberg and Cousins, 2006).

With medicinal plants, temporary emersion tissue culture systems for *Artemisia judaica* showed higher rates of shoot multiplication and fresh weight accumulation compared to culture systems with physical support such as agar or paper bridges; material derived from temporary immersion bioreactors possessed strong antioxidant capacity following extended growth in a greenhouse (Liu et al., 2004). A temporary emersion system for *Scutellaria baicalensis* was shown to produce large amounts of biomass and greater metabolite than most of the other tissue culture systems evaluated (Zobayed, et al., 2004b). A study of various in vitro systems for *Hypericum perforatum* indicated that temporary emersion systems were not ideal and performed more poorly than other systems with regard to biomass accumulation and metabolite accumulation (Zobayed et al, 2004a). A temporary immersion system for *Artemisia annua* L. was found to produce artemisinin in quantities intermediate between continuous immersion and nutrient mist

bioreactors; dry weight accumulation was also intermediate between the other two bioreactor types (Liu et al., 2003).

IV. Enhanced Production of Secondary Metabolites

Lowering laboratory costs and improving yield are both likely steps in developing industrial scale medicinal plant bioreactor systems. During in vitro studies of secondary metabolism, many methods for "upregulating" metabolism have been explored with promising results. Unfortunately, few of these methods have seen wide scale exploration in whole plant culture applications. Several major strategies have been explored for upregulation of metabolism including precursor feeding, hormonal signaling, elicitation, and nutrient stress.

Jasmonic acid is frequently produced by plants as a result of pathogen presence, wounding, or insect attack. This statement was supported by Blechert et al. (1995) who found increased jasmonic acid content in differentiated leaf tissue and cell suspension cultures when exposed to insect attack and fungal cell wall preparations, respectively. Plant defense hormones such as jasmonates have been shown by many scientists to induce secondary metabolite production (Gaisser and Heide, 1996; Gundlach et al., 1992; El-Sayed and Verpoorte, 2002; Zabetakis et al., 1999). Jasmonic acid was found to stimulate storage organ formation in garlic with a 10 μM solution eliciting optimum results (Kim et al., 2003). Phenylalanine ammonia lyase (PAL), a key enzyme that links primary metabolism to secondary metabolism by serving as a catalyst in the deamination

of phenylalanine, was found to be activated by exposure to methyl jasmonate (Sharan et al., 1998). This is considered to be the first step in the shikimic acid pathway.

Precursor feeding has also been used for medicinal chemistry production. Phenylalanine, tyrosine, and tryptophan are the aromatic amino acid products of the shikimic acid pathway (Schmidt and Amrhein, 1995). The precursors in phenolic biosynthesis are the products of the shikimic acid pathway (Bruneton, 1999). Phenylalanine is a precursor for most phenolic compounds in plants and has been successfully used to induce metabolite production in vitro in many different plant systems (Ibrahim, 1987; Fett-Neto et al., 1994; Petersen, 1999).

Fungal elicitation has seen encouraging results as an inducer of secondary metabolism. Exposure of basil to chitosan caused significant increases in medicinal compound production (Kim et al., 2005). Fungal mycelium homogenate caused production of antifungal monoterpenes in *Piqueria trinervia* (Saad et al., 2000). Chitosan hydrolysates increased paclitaxel production in cell cultures of *Taxus canadensis* (Linden and Phisalaphong, 2000). Other less common methods have been attempted successfully as well. Staniszewska et al. (2003) successfully used BION® (benzo(1,2,3)-thiadiazole-7-carbothionic acid *S*-methyl ester) and heat killed *Enterobacter sakazaki* to induce metabolite production using multiple in vitro culture methods. Yeast extracts have been used alone and in conjunction with jasmonates to enhance secondary metabolite production from *Silybum marianum* (Sanchez-Sampedro et al., 2005).

Exposure of plant materials to the amino acid L-proline has been shown to increase carbon flow through the pentose phosphate pathway and alter energetic

metabolism to increase production of products from the shikimic acid pathway and phenyl propanoid pathway. This process has been shown to upregulate metabolite production in many plant species. Proline and analog hydroxy proline have been shown to cause increases in phenolic and rosmarinic acid contents of thyme (Kwok and Shetty, 1998). Similar results were found for oregano (Yang and Shetty, 1998). Duval and Shetty showed that phenolics and antioxidant activity were induced by proline increases (2001).

Low nitrogen nutrition has been shown to lead to further metabolite production. Flavonol accumulation increases have been documented in plants where nitrogen is deficient (Stewart et al., 2001). Nitrogen deficiency led to increased levels of phenolics and increased activity of phenylalanine ammonia lyase (Kovacik et al., 2007). In tobacco, nitrogen deficiency led to a shift from nicotine production to production of more carbon rich metabolites (Fritz et al., 2006).

V. Turmeric (*Curcuma longa* L.) as a Model System

Botany and Horticulture

Turmeric is a short stemmed perennial that grows to be 0.5-1 m tall. Leaves are approximately 1.2 m in length and are oblong-lanceolate with long petioles that make up about half of the leaf length. Leaves are roughly 8 cm wide at the widest point. This monocotyledonous plant is a triploid, and as a result, it is incapable of undergoing sexual reproduction. Sterile flower spikes are about 12 cm in length. Turmeric flowers are showy, fragrant, and white or yellow in color. Ovate or cylindrical rhizomes that are shortly branched proliferate at the base of the plant. An aromatic odor emanates when

rhizomes are cut. Rhizome internal color ranges from yellow to orange (Kapoor, 1990; Parrotta, 2001).

Turmeric is a sterile triploid necessitating repeated vegetative propagation of the subterranean rhizome. The divisions are planted in the field and allowed to grow for two years. Field culture is reduced to 12 months when the entire mother rhizome is planted (Wardini and Prakoso, 1999). These methods for production and propagation necessitate the retention of 10% of the material produced for planting the following year. India produces about 635,950 t/year (Ravindran, 2007) or about 80% of the world supply of commercial turmeric. This tonnage is produced over an area of approximately 175,190 ha (Ravindran, 2007). Though the plant is grown throughout India, the majority is produced in Madras, Bengal, and Bombay (Kapoor, 1990). The rhizomes of the plants are harvested and dried before shipment around the world (Bruneton, 1999). There is no breeding or true seed. All field planting must be done with stock that is prone to carry soil borne diseases. Replanting subterranean divisions in tropical soils spreads fungal and bacterial diseases including bacterial wilt, *Pythium* rot, and *Fusarium* yellows, along with various leaf spots and blights from nurseries to production fields.

Traditional Uses and Products

Curcuma longa L. (turmeric) is a plant that has been used in traditional medicine for thousands of years. Turmeric is used as a spice component of curry powder in the ethnic cuisine of southern and eastern Asia. This plant is also used to make a yellow textile dye. The curcuminoids are the dyeing agent found in turmeric. Additionally, turmeric is used as a commercial starch. Curcuminoids in the US food industry are

labeled as natural yellow dye number four and are used to color many products including mustard. Ayurvedic medical systems have different uses for both fresh and dried preparations of turmeric with dried powders being used to treat distinctly different ailments from pastes or plant juices (Kapoor, 1990; Parrotta, 2001). This plant has recently garnered the interest of the wider research community as its medicinal properties have been discovered and studied (Carolina et al., 2003). Unfortunately, recent scientific examinations have found that methods designed to detect adulteration of turmeric powders are lacking. These researchers have called into question the quality of commercially produced turmeric and suggested alternative means for product quality control (Sasikumar et al., 2004).

Turmeric and ginger, *Zingiber officianlis*, have similar morphology, culture, and phytochemistry, but turmeric has an antioxidative activity approximately ten times higher than that of ginger (Premavalli, 2005). In turmeric, the oleoresin yield was 10.5% at 19 weeks, 10% at 21 weeks, and 8% by 39 weeks although economic harvest is done with one and two year old rhizomes. Dry matter increased from 19 to 39 weeks (Balakrishnan, 2007).

Constituents and Activities

Nahar and Sarker (2007) noted 80 metabolites that have been identified from turmeric. Hundreds of activities and modes of action for these metabolites have been discovered and characterized as well (Sarker and Nahar, 2007). As a result of its many constituents and activities, much research has been done on this plant.

Many activities of turmeric constituents have been elucidated in the recent past, and some of them have shown activities against plant pathogens. Kim et al. (2003) found that turmeric rhizome extracts possessed potent fungicidal activities against five phytopathogenic fungi (including *Phytophthora infestans*); curcumin and turmerone were implicated as participants in this activity. One study demonstrated that 15 dermatophyte isolates could be inhibited by turmeric oil though their growth was not inhibited by curcumin; turmeric oil also inhibited four pathogenic fungi while curcumin was ineffective (Aplsarlyakul et al., 1995). Chowdhury et al. (2000) found that curcuminoids, curcuminoid derivatives, and turmeric oil caused moderate growth inhibition and mortality in *Schistocerca gregaria* and *Dysdercus koenigii*, which indicates moderate insecticidal properties.

Five turmeric constituents (three curcuminoids and two other diaryl compounds) have shown promise for treatment of Alzheimer's disease through their assayed ability to protect PC-12 cells from beta-amyloid insult better than Congo red (Park and Kim, 2002). Beta amyloid plaque accumulation is acknowledged as a potential cause of Alzheimer's disease, and Congo red is used as a positive control for assaying compounds for prevention of plaque accumulation.

Yu et al. (2002) found that turmeric attenuates the activity of monoamine oxidase (an enzyme involved in monoamine neurotransmitter metabolism) as do many other accepted antidepressants (Wouters, 1998) and postulated that turmeric might help mediate the effects of immune-associated depression. Curcumin has shown promise for possible use in treatment of multiple sclerosis (Natarajan and Bright, 2002).

Turmeric has been shown to alleviate symptoms of many digestive and respiratory related problems with the proposed mechanism of action being related to relaxation of hyperactive tissues within these systems through calcium channel blockage (Gilani et al., 2005). Turmeric has been proposed as a treatment for gallbladder disease (Moga, 2003). Harsha et al. (2002) found that turmeric rhizomes are used to treat urinary tract infections by the Kunabi Tribe of India. Turmeric extracts were found to inhibit inflammation and joint destruction associated with arthritis (Funk et al., 2006).

Curcumin was shown to lower blood cholesterol in both diabetic rats and rats fed a high cholesterol diet as compared to controls indicating its potential to be used to fight atherosclerosis (Babu and Srinivasan, 1997). Sharma et al. (2006) showed that curcumin was capable of protecting kidneys from diabetic nephropathy as a result of increased blood glucose and hypothesized that the antioxidative qualities of this compound are likely responsible for this activity.

A major activity of study is the significant antioxidant capability of turmeric chemical constituents. Much work has been carried out on the antioxidant activities of compounds derived from turmeric rhizomes. The curcuminoids [curcumin (1,7-bis(4-hydroxy-3-methoxyfenil)-1,6-heptadiene-3,5-dione], demethoxycurcumin, and bisdemethoxycurcumin) are the major antioxidant compounds of turmeric (Figure 1.1). The curcuminoids can undergo keto-enol tautomerism (Figure 1.2) at the β-diketone group in the central carbon chain. The enol form of curcumin creates a conjugated system that allows the carbon backbone to be stabilized by resonance. The β-diketone moiety is believed to be involved in antioxidant and tumor preventive activities of this group of

compounds (Sugiyama et al., 1996). The benzene rings present in the compounds are also participants in antioxidant capacities due to their stability even when exposed to radicals.

In addition to the curcuminoids, there are many other compounds (some of them in the volatile essential oil), which possess antioxidative properties including: γ-terpinene, ascorbic acid, β-carotene, β-sitosterol, caffeic acid, campestrol, camphene, dehydrocurdione, eugenol, p-coumaric acid, protocatechuic acid, stigmasterol, syringic acid, turmerin, turmeronol-a, turmeronol-b and vanillic acid (Duke, 2004). Selvam et al. (1995) isolated a heat stable antioxidant protein simply called turmeric antioxidant protein. Another study found curcumin to be a potent quencher of singlet oxygen species (Das and Das, 2002).

Curcumin is thought to suppress cancer development by helping inhibit enzymes which lead to tumor production (Surh, 2002). Curcumin also prevents cancer along with inflammation by inducing production of enzymes used to detoxify electrophilic species produced in lipid peroxidation (Piper et al., 1998). Verma et al. (1997) showed that curcumin inhibited the growth of human breast cancer cells. Methanolic extracts of turmeric were found to inhibit cyclooxygenase and nitric oxide synthase, enzymes responsible for production of prostaglandins and nitric oxide; these two species are often implicated in many detrimental processes such as inflammation and carcinogenesis (Hong et al., 2002). Additionally, experiments suggest that turmeric inhibits production of tumors as a result of exposure to benzo(a)pyrene (Thapliyal et al., 2002). Chakravarty and Yasmin (2005) found that turmeric extracts induced lymphocyte production while causing apoptosis of carcinoma cells following membrane destruction. Curcumin has

been shown to help limit cell variability and proliferation of skin cancer by inducing apoptosis in melanoma cells (Siwak et al., 2007). Turmeric rhizomes are used externally to lessen and heal rash symptoms such as itching, small breaks in the skin, irritation, and inflammation (Wardini and Prakoso, 1999).

Tissue Culture

In vitro propagation techniques have facilitated the dispersal of clean planting stock by crown divisions of leafy shoots. More recently, microrhizomes were found to develop in vitro in liquid medium with increased sucrose levels found to correlate with rhizome size. Larger microrhizomes were found to be capable of survival in the field without any acclimatization (Shirgurkar et al., 2001; Salvi, et al., 2002). Nadgauda et al. (1978) developed an agar based culture system using repeated excision of rhizome vegetative buds to create large numbers of turmeric plants (approximately 200,000 per year for a slower dividing variety and 800,000 per year for a faster dividing variety) with field survival rates of approximately 65%. A later study obtained nearly 100% survival of tissue cultured material following planting in the field (Balachandran et al., 1990). Nadgauda and Mascarenhas (1986) characterized a tissue culture method for screening *Curcuma* varieties for high curcumin production through analysis of what were believed to be rhizome primordia after callus regeneration and before growing larger propagules suited to field planting. Tyagi et al. (2007) demonstrated that lower cost medium components such as lower grades of sucrose and cheaper gelling agents can be used for germplasm storage with good results.

Active storage organs develop as part of differentiated in vitro whole plant culture of *Curcuma longa* in a simple intermittent immersion bioreactor (Adelberg and Cousins, 2006). Potent antioxidants were produced in vitro in the microrhizomes (Cousins et al., 2007). The drying process has a negative effect on the antioxidant capabilities of turmeric extract. Heat processing (boiling and or pressure cooking) of turmeric had significant negative impact on curcumin content of tissue with ten minutes of pressure cooking decreasing curcumin concentration by approximately half (Suresh et al., 2007). The antioxidant potential of turmeric microrhizomes in this system is quite strong compared to commercially procured rhizome powder (Cousins et al., 2007). Fresh microrhizome extracts were more potent than dried microrhizome extracts, which were more potent than commercially prepared dried turmeric powder.

Objectives and Rationale

Potential use of plant and organ culture in fostering medicinal plant research and compound production has great potential to significantly expedite commercialization. The inherent variability in phytochemical composition of herbal products as demonstrated by Bauer and Tittle (1996) can be controlled through the use of plant and organ culture as discussed in this review. In vitro systems can also be adapted for specific upregulation of medicinal compounds for production of higher value material.

There are two basic methods for enhanced metabolite production in plant or organ culture systems. One, maximize or improve active biomass production through manipulation of basic media components and vessel system types. Two, manipulate plant

metabolism through one of many strategies to increase metabolite concentration in the plant material. These two points will serve as the objectives for the remainder of this thesis work as we explore these two methods for secondary chemistry production in turmeric.

In an attempt to further study the first point, we will conduct a time course experiment that will explore biomass accumulation over time in vitro. This experiment will yield a better understanding of the accumulation of biomass in response to different media formulations. There are many strategies that can be employed in the development of the second point that should be explored in medicinal plant systems for increased metabolite production. We will attempt upregulation of secondary metabolite production using hormone application with methyl jasmonate, anabolic process modulation with proline, defensive signaling with chitin, and nutrient status modification.

Knowledge of these two points, biomass and upregulation with regard to turmeric will allow for development of better systems for production of secondary metabolites in turmeric. In the future, exploration of various other plant and organ culture systems must investigate these two main points in order to enhance medicinal chemistry production with in vitro systems.

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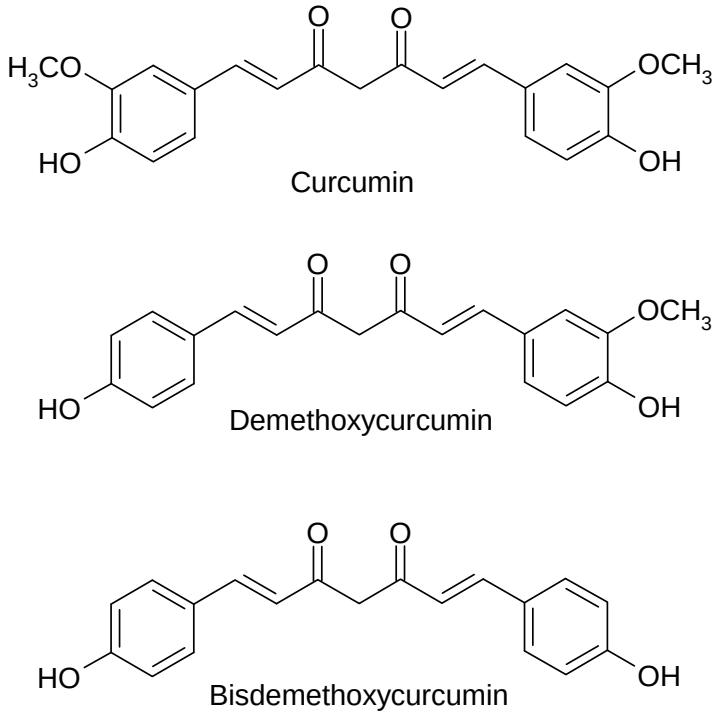

Figure 1.1. The structures of three curcuminoids.

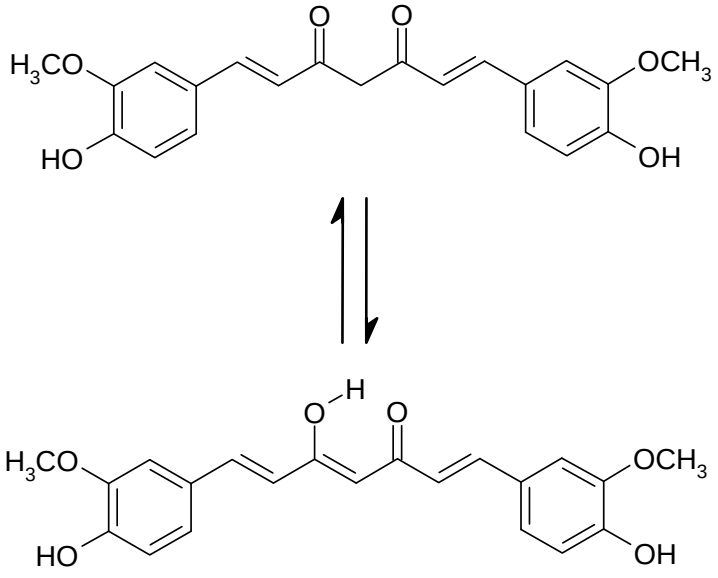

Figure 1.2. Keto (top) and enol (bottom) forms of curcumin.

CHAPTER TWO

SHORT-TERM AND LONG-TERM TIME COURSE STUDIES OF TURMERIC (*CURCUMA LONGA* L.) MICRORHIZOME DEVELOPMENT IN VITRO

Abstract

Turmeric (*Curcuma longa* L.) plantlets were cultured in MS liquid medium with 6% sucrose. Microrhizome development was observed in the presence of methyl jasmonate (MeJa) (0, 5 and 16 μM) and benzyladenine (BA) (0, 0.32 and 1μM). Leaf, root, rhizome growth, and sugar use were measured weekly for 6 weeks in small vessels (180 ml) and four times in 23 weeks in larger vessels (2.5 L). MeJa reduced leaf, root, and rhizome biomass. BA had a positive effect on biomass accumulation. Microrhizome mass increased at a linear rate during 6 weeks of culture while roots and leaves accumulated biomass at an exponential rate. Sugar use correlated nearly directly to whole plant dry weight (DW) in the short and long-term experiments. Microrhizomes became a larger fraction of whole plant DW as plantlets aged. After 6 weeks, about 1.8 g of microrhizome DW per liter of media had been produced (in both time courses), and after 23 weeks, about 26 g of microrhizome DW per liter of media had been produced. Secondary rhizomes were first observed at 14 weeks, and most plants had them by 23 weeks. A method for rhizome production in a long-term culture system was described. The linear relationship between DW and sugar use will be useful in the eventual development of a model for sugar use to biomass to secondary metabolite production.

Abbreviations: Benzyladenine – BA; Dry Weight – DW; Fresh Weight – FW; Methyl Jasmonate – MeJa; Murashige and Skoog – MS; Plant Growth Regulator – PGR; Relative Dry Weight (DW/FW) – RDW

Introduction

Turmeric (*Curcuma longa* L.), an herb that has been used in Indian cuisine and traditional medicine for centuries, has gained reinvigorated interest due to its significant medicinal potential. Turmeric is used as a dye, spice, and industrial starch in commercial applications. A yellow textile dye is made from the characteristic yellow-orange curcuminoids found in the rhizomes. Turmeric is a component of curry powder and is popular in the countries of southern and eastern Asia. Ayurvedic medical systems have different uses for both fresh and dried preparations with dried powders being used to treat distinctly different ailments from pastes or plant juices (Kapoor, 1990; Parrotta, 2001).

Roughly 80% of the world's supply of commercial turmeric is produced in India (about 635,950 tons/year) (Ravindran, 2007). Turmeric plants are sterile triploids and must be maintained by repeated vegetative propagation of rhizomes. The divisions are field planted and allowed to grow for one or two years depending on size and quality of propagules (Wardini and Prakoso, 1999). Replanting subterranean divisions in tropical soils spreads fungal and bacterial diseases. Rhizomes are slow to multiply, and yields range from 15-20 tons per hectare (Balachandran et al., 1990).

Turmeric has been clonally propagated in vitro through shoot (Nadgauda et al., 1978) and rhizome callus induction (Yasuda et al., 1988). More recently, microrhizomes

were found to develop in vitro in liquid medium with increased sucrose levels correlated with rhizome size, and larger microrhizomes were capable of survival in the field without any discreet acclimatization procedure (Shirgurkar et al., 2001; Salvi et al., 2002). Microrhizomes were also found to produce secondary metabolites with antioxidant activity equaling or surpassing commercial dried powdered rhizome preparations of field-grown plants (Cousins et al., 2007).

There are many reports on methods to induce microrhizome development in *Curcuma*. Most researchers have used liquid MS medium modified with benzyladenine (BA), a common synthetic cytokinin. Other plant growth regulators, increased sucrose concentration, and modified environmental conditions have also been employed in attempts to promote microrhizome development. Raghu (1997) produced microrhizomes on liquid MS medium supplemented with 1.33 μM BA, 0.54 μM 1-napthylacetic acid (NAA), 1.95 μM ancymidol, and 10% sucrose. Sanghamitra and Nayak (2000) produced microrhizomes on liquid MS medium with 13.32 μM BA, 6% sucrose, and a 4 hour photoperiod. Shirgurkar et al. (2001) found that 4.4 μM BA allowed microrhizome development while high levels (35.2 μM) of BA could completely inhibit microrhizome formation in turmeric. Intermediate BA levels also caused decreased microrhizome development. Sunitibala et al. (2001) grew rhizomes on liquid MS medium supplemented with 0.54 μM NAA, 4.65 μM kinetin, and 8% sucrose. In our laboratory's turmeric micropropagation medium (liquid MS medium modified with 6% sucrose and 1 μM BA), increased vessel size (from 180 ml jars to 6L plastic vessels) led to increased turmeric plant size (though larger vessels with agar media did not increase plant size), and

rhizomes functioned as storage organs (Adelberg and Cousins, 2006). Adding 3.2 μM ancymidol to our turmeric micropropagation medium reduced fresh weight by about 10% without increasing rhizome mass. We also tested 2%, 4%, 6%, and 8% sucrose media with 1 μM BA and found that solute partitioning to rhizomes was greatest in 6% sucrose media (Adelberg and Cousins, 2007).

In vitro application of jasmonic acid has been reported to increase the size and numbers of storage organs in sugar containing medium in many bulb forming species (Norjiri et al., 1992; Ravinkar, et al., 1993; Santos and Salema, 2000; Kim et al., 2003). Jasmonic acid production is often induced in plants as a result of pathogen presence, wounding, or insect attack; its concentration increased in differentiated leaf tissue and cell suspension cultures when exposed to insect attack and fungal cell wall preparations (Blechert et al., 1995). Many reports state that jasmonate can induce secondary metabolite production (Gaisser and Heide, 1996; Gundlach et al., 1992; Zabetakis et al., 1999). MeJa altered expression of phenylalanine ammonia lyase (Sharan et al., 1998) in the shikimic acid pathway (Schmidt and Amrhein, 1995) – a pathway responsible for production of many phenolic secondary metabolites.

The quantity of tissue and concentration of metabolites in that tissue both influence metabolite yield. As a result, the most effective metabolite production is in systems that produce large amounts of biomass that contain high concentrations of active metabolites. The current study will further characterize the growth of turmeric plants in vitro over long periods of time while observing the effects of plant growth regulators (BA and MeJa) on the development of plant organs in large and small vessels. Correlations

between sugar use and whole plant and microrhizome growth and development will be drawn. Analysis of secondary metabolite activities from similar studies has been conducted and will be discussed in Chapter 3.

Materials and Methods

Plant material

In vitro stock plants. Turmeric accession L35-1 was obtained from the University of Arizona Southwest Center for Natural Products Research and Commercialization. Stage I was prepared by dissecting the quiescent shoot tips from rhizomes, immersing them in full-strength commercial bleach (Clorox™, 5.25% sodium hypochlorite) for 30 seconds, allowing them to air dry on the hood surface, and placing them in PGR free liquid modified MS medium (Murashige and Skoog, 1962) that included: 170 mg NaH_2PO_4, 100 mg myo-inositol, 0.25 mg nicotinic acid, 0.25 mg pyridoxine hydrochloride, 0.05 mg thiamine hydrochloride, and 30 g sucrose per liter. Medium pH was adjusted to 5.7 before being dispensed. Stage I was conducted with 2 ml of medium in flat-bottomed shell vials (diameter 23 mm). Stage II cultures were maintained for at least 6 months by subculture every six weeks in MS media of the above formulation but with 1 μM BA and 60 g sucrose per liter media. Subculture was conducted in 180 ml glass jars containing 30 ml of liquid tissue culture medium. Material was cultured on an orbital shaker (100 rpm) with 25 to 35 μmol s^{-1} m^{-2} photosynthetically active radiation provided by cool white fluorescent tubes with 16 h d^{-1} photoperiod maintained at 24 $\pm$ 2 °C at a rate of 3-4 buds per jar.

Prior to experiment start, a final round of stage II subculture was conducted. Buds were taken from the normal 1 μM BA media and placed into one of three BA levels (0, 0.32, or 1 μM) for one six-week cycle prior to commencement of the PGR factorial time course experiment. On beginning the experiment, buds conditioned to the 0 μM BA level were utilized to make the 0 μM BA experimental units, buds conditioned to the 0.32 μM BA level were used to make the 0.32 μM BA experimental units, and buds conditioned to the 1 μM BA level were used to make the 1 μM BA experimental units. This was done to minimize carryover of BA into the experimental phase.

PGR factorial in time course experiments

Short-term time course. BA concentrations (0, 0.32, and 1 μM) and MeJa concentrations (0, 5, and 16 μM) were arranged in 9 treatment factor combinations (3 x 3). Shoot and root systems were removed, and two buds were placed in each jar with 33 ml of medium. Material was cultured on an orbital shaker (as above). Four predetermined jars from each treatment factor combination were harvested destructively each week for six weeks. Residual sugar concentration in expended liquid media was determined with a refractometer (Model # N-10E – ATAGO, U.S.A., Inc., Bellevue, WA). Plants were dissected, and leaves, rhizomes, and roots were grouped together prior to FW and DW determination. Plant dry weight (DW) was recorded after drying the contents of each vessel individually in paper envelopes at 70 ºC for 48 h.

Long-term, fed-batch time course. Large rectangular vessels (approximately 2.5 L), (Liquid Lab Vessels, Southern Sun Inc., Hodges, SC; Adelberg and Simpson, 2004),

were agitated on a thin-film rocker system (Figure 2.1) (Adelberg and Cousins, 2006) that produced a 1 cm pitch every 15 minutes (with a 1 rpm cam). Each vessel contained 200 ml of medium of the same formulation and treatment combinations as used in the short-term time course. Plants were conditioned to BA concentrations (0, 0.32, or 1 μM) prior to experiment start. Vessels were inoculated with plant material from one six-week old jar (13-17 plantlets trimmed free of roots) grown at appropriate BA concentration. Two vessels at each of the 9 BA and MeJa treatment-factor combinations were harvested at three time points (6, 14, and 23 weeks) over the 23 week time course. The harvest method was the same as that used for the short-term time course. According to our fed-batch process, media was added to each remaining vessel following the harvest date to return the media volume to approximately 200 ml and sucrose concentration to approximately 6%. Following 6 weeks in culture, two vessels from each treatment condition were harvested as above. Approximately 75 of the 200 ml of medium remained in the vessels at the end of 6 weeks of culture. This residual medium was 3% sucrose compared to the initial 6% sucrose. Therefore, 125 ml of 8% sucrose medium was added to the unharvested vessels to replenish the medium to approximately 200 ml of 6% sucrose medium. After 14 weeks, 90 ml of medium containing 1.2% sucrose remained in each vessel. Therefore, 110 ml of 10% sucrose medium was added to replenish the medium to 200 ml with 6% sucrose. The final group of vessels was harvested at 23 weeks.

Field-grown plants. Plantlets grown on 1 μM BA media in vitro were acclimatized in moist soilless mix (Fafard 3B, Fafard Inc., Anderson, SC) in 1204 plant

cell packs under intermittent mist (6 s of mist every 6 minutes during day) for 3 weeks. Subsequently, they were hand-watered in a conventional greenhouse for another 3 weeks. Acclimatized plants were then transferred to a natural soil bed in a covered, heated field structure (Specialty Plants House, Fort Valley State University, Fort Valley, GA). Flood irrigation was provided as necessary, and plants were grown from March to December in 2005 (one growing season).

Experimental design and analysis

The experiment was a completely randomized design for the 9 PGR treatment conditions (3 levels of BA and 3 levels of MeJa arranged as a 3 x 3 factorial) with vessels being the unit of replication (4 replicates of small jars in the short-term time course and 2 replicates of large vessels in the long-term time course). One set of vessels was harvested on each of 6 harvest dates for jars and 4 harvest dates for large vessels. The data were analyzed separately for short-term (small vessels) and long-term (large vessels) studies. For the long-term time course, PGR data was pooled as determined to be appropriate by ANOVA with $\alpha = 0.05$. Short-term time course fits were separated using the log likelihood ratio test with $\alpha = 0.01$. Best fits of selected response variables are presented. Statistical analysis was performed using JMP 3.2.6 (SAS Inst., Cary, NC, USA) and SAS Version 9.1 (SAS Inst., Cary, NC, USA), and graphics were created with Statistica Version 7.1 (Statsoft Inc., Tulsa, OK, USA).

Results

Short-term time course in small jars for six weeks

Growth of leaf, root and rhizome biomass (FW and DW) is shown for the 9 combinations of growth regulators over the six weekly observations (Table 2.1). There was a definite dichotomy between linear increases in mass of rhizome and exponential increases in mass of leaves and roots.

Plantlets accumulated biomass most rapidly in the absence of MeJa in our standard medium containing 1 μM BA. Plantlet growth was negatively impacted by the presence of MeJa (Figure 2.2a). Plant DW accumulation was slowed by 16 μM MeJa as was rhizome growth (FW and DW accumulation). Leaf FW (Figure 2.2b) and DW growth over time was negatively impacted by MeJa with the 5 and 16 μM levels yielding reduced leaf mass. Root FW (Figure 2.2c) and DW displayed similar results with decreased root growth at 16 μM MeJa. Increases in MeJa concentration lowered leaf, root, and plant biomass in 1μM BA media (Figure 2.3).

In the lower BA concentration (0.32 μM), plant, root and leaf FW accumulation (Figure A-1) were negatively affected by 16 μM MeJa while plant DW and rhizome biomass (FW or DW) were unaffected by MeJa. MeJa effects on root and leaf growth were more varied than we observed in 1 μM BA. In the absence of BA, MeJa had no detectible effect on plantlet growth – leaves, roots and rhizomes (FW or DW) (Figure A-2). Only shoot FW was negatively affected by 16 μM MeJa.

Lowering BA concentrations to 0 μM or 0.32 μM did not promote rhizome development. Shoot, root and whole plant growth were not enhanced either. The effects

of lowered BA concentrations were only detectable in the absence of MeJa, and plants grown in media with 1μM BA grew best as measured by total FW, rhizome FW (Figure 2.4; Figure A-3) and rhizome DW.

Microrhizome FW and DW increased at a linear rate over the short-term time course (Figure 2.5; Figure A-4). RDW [relative dry weight (DW/FW)] of microrhizomes was approximately 0.10 in small jars. Leaves and roots had lower RDWs than rhizomes (data not shown). MeJa caused an increase in rhizome harvest index (rhizome DW/total DW) after 6 weeks of growth – 45% (0 μM MeJa), 51% (5 μM MeJa), 57% (16 μM MeJa) for DW. BA had no effect on this factor.

Long-term fed-batch time course in large vessels for twenty-three weeks

MeJa and BA concentrations did not influence growth (whole plant FW and DW) in the long-term fed-batch time course in larger vessels with media supplementation (data not shown). Whole plant FW and DW increased over the entire 23 weeks (Figure 2.6a), but the rate of FW gain declined slightly around 15 weeks. The rate of rhizome DW accumulation increased over 23 weeks (Figure 2.6b) as did the RDW of the rhizome tissue (Figure 2.6c). In the long-term time course, the RDW was approximately 0.10 during the first 6 weeks (similar to jars) but increased to 0.12 and 0.16 by weeks 14 and 23, respectively. Harvest indexes increased over the course of the experiment from 38% at 6 weeks to 47% at 14 weeks and 60% at 23 weeks.

At 14 weeks, secondary rhizomes became evident upon peeling back the leaves and roots covering the rhizomes. These new structures radiated from primary rhizomes in

a few of the specimens (Figure 2.7a). By week 23, leaf blades and roots were visibly atrophied and rhizome enlargement was evident (Figure 2.7b, c). When roots and leaves were pulled away, most rhizomes were swollen with secondary rhizomes present.

By week 23, about 26 g of rhizome DW had been produced per liter of media (based on 435 ml per vessel). The 2.5 L vessels were fed 33 g sucrose, and over 32 g of that sucrose was consumed by the plant material. Over 23 weeks, 11.3 g of rhizome DW and 19 g of total DW were accumulated per vessel. Therefore, about 59% of the sugar mass was incorporated as DW. Rhizome RDWs were 0.16 at 23 weeks, and 0.63 g rhizome DW was produced per plant. When vessels were opened, a pleasant aroma was observed. Also, rhizomes displayed the yellow color characteristic of curcuminoid phenolics when they were cut.

Relationship between sucrose and biomass

There was a nearly direct relationship (Figure 2.8) between sugar use and DW produced. Calculations showed that a 1 g increase in tissue DW required approximately 1.8 g of sugar from the medium. Therefore, approximately 59% of the sucrose mass was incorporated into the plantlet as DW with the remainder being released as CO_2 or lost as a result of other plant processes such as phenolic exudation or cell sloughing. In vitro plant cells generally exhibit a conversion efficiency of approximately 50%, meaning that half of the organic carbon taken up from the media is converted to cell DW in bioreactor systems (Curtis, 1999). We may conclude that the turmeric plantlets grown in liquid MS medium were of similar efficiency. For each 2 g of sugar used, 0.73 g of plant DW and

0.37 g rhizome DW were produced. After 6 weeks, tissue from jars was of similar quality and size to that generated in large vessels over the same time period (Figure 2.9).

Field grown system

After 23 weeks of growth in soil, field grown rhizomes from clone L35-1 had an RDW of 0.24. Field grown plants produced 30 g to 60 g rhizome FW each. These rhizomes had deep yellow-orange coloration and released aromatic odors when cut. Field grown plants had greater rhizome mass than jars would have produced in the same period of time (determined through extrapolation from 6 to 23 weeks based on linear rates of microrhizome mass increase in jars). In the long-term time course, microrhizomes had an increasing rate of DW accumulation (Figure 2.6b) and appeared more like field grown tissue at the final harvest. In vitro microrhizomes at 23 weeks were still growing much slower than field plants. Sucrose concentrations in the large vessels observed at weeks 6, 14 and 23 were 3%, 1.2% and 0.5% respectively. Our research has shown that plants grow best at media sucrose concentrations approximating 6%. As a result, we believe that the plants spent a great deal of time at suboptimal sucrose concentrations. This caused some portion of the difference in growth rates between the field produced and in vitro derived tissues. An optimized fed-batch system would more closely approximate the growth rates seen in field grown turmeric plants.

Discussion and Conclusions

Short-term time course in small jars for six weeks

The growth of vegetative tissues typically has a lag phase, followed by an exponential rate of increase until a senescent phase begins due to limitations in resources. The leaves and roots in the short-term time course showed this characteristic behavior but did not extend to a point of growth limitation by week 6. MeJa delayed the onset of the exponential growth phase with leaves and roots. However, rhizomes did not enter the exponential phase of growth during the short-term time course.

This experiment also indicated that turmeric had inadequate endogenous cytokinin for optimum rhizome growth and disproves our earlier belief that the 1μM BA concentration in our micropropagation medium was too high for optimal rhizome development.

Long-term fed-batch time course in large vessels for twenty-three weeks

Long-term growth was made possible through fed-batch media supplementation over the time course and enabled formation of functional rhizomes with secondary branching. Also, over time, larger percentages of the total plant biomass were contained in the active storage organ – the rhizome. Despite the addition of fresh medium, leaves and roots started to atrophy and began the senescent phase of their growth cycle (Figure 2.7). Rhizome dry matter accumulation increased at the expense of leaf and root growth as rhizomes became competitive sinks. The finding that turmeric plants have higher RDW in the rhizome than in the other organs (data not shown) indicates active

accumulation of carbohydrates in storage organs and is consistent with prior observations of turmeric microrhizomes in vitro (Adelberg and Cousins, 2006; 2007). The growth rates of in vitro rhizomes were still lower than growth rates in field culture.

The relationship between sugar use and DW for the short-term time course was similar to the relationship between sugar use and DW for the long-term time course (Figure 2.8). A refractometer can be useful to determine when nutrient supplements are needed in a system where sugar is the limitation to growth. Similar factors may describe relationships between secondary metabolite production and DW. Sucrose concentration could be utilized to monitor biomass during production and prior to harvest. This method of quality control and reliable yield estimation would serve to create a repeatable and easily standardized culture method.

Conclusions

Biomass accumulation curves for the leaf, root, and whole plant approximated the lag phase, exponential growth phase, and beginning of linear growth phase portions of the S-shaped curves similar to those seen in cell cultures (George et al., 2008). Rhizomes, however, grew at linear rates. MeJa discouraged biomass accumulation while BA had the opposite effect by encouraging organ development. DW was correlated to sugar use and the regression equations from the short-term and long-term time course experiments were nearly identical. As a result, we find that there is the potential to reliably extrapolate beyond a short six week growth period to estimate the amount of DW that would be accumulated over a much longer period of time – in the case of this experiment, 23

weeks. This finding also alludes to a production scheme where sucrose concentration is used to estimate biomass production during tissue growth.

Large amounts of microrhizome tissue were effectively produced over the course of this study. Such plant materials from a similar production system could be used as propagation stock for development of robust field plants or for rapid dissemination of germplasm. The authors have acclimatized hundreds of similar plants from large vessels to greenhouse conditions without any loss.

Alternatively, this production system could be utilized for production of secondary metabolites for chemical analysis, other experimentation, or direct usage. The quality and concentration of secondary metabolites produced by plants grown in this system were addressed in another experiment (the focus of Chapter 3).

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Table 2.1. Biomass (g per 180 ml vessel) at each harvest period for the 9 BA and MeJa treatment combinations. Best fits for changes in biomass over time are denoted as either exponential (E) or linear (L).

	Treatment (μM)		Weeks						
Response	BA	MeJa	1	2	3	4	5	6	Fit Type
FW Shoot	0.00	0	0.202	0.425	1.251	1.839	1.916	3.798	E
	0.00	5	0.121	0.476	0.658	1.606	2.121	1.894	E
	0.00	16	0.091	0.420	0.379	0.327	1.144	2.718	E
	0.32	0	0.174	0.602	1.071	1.890	2.535	3.733	E
	0.32	5	0.205	0.250	0.648	1.281	1.851	3.500	E
	0.32	16	0.148	0.137	0.328	0.819	0.856	1.516	E
	1.00	0	0.148	0.325	1.188	2.052	1.895	3.560	E
	1.00	5	0.113	0.202	0.746	0.640	1.319	2.842	E
	1.00	16	0.095	0.215	0.236	0.581	0.921	1.624	E
FW Root	0.00	0	0.018	0.078	0.287	0.994	1.153	2.464	E
	0.00	5	0.025	0.098	0.323	1.013	1.695	2.601	E
	0.00	16	0.044	0.135	0.121	0.210	0.971	2.433	E
	0.32	0	0.051	0.212	0.391	1.169	2.184	4.200	E
	0.32	5	0.027	0.104	0.206	0.853	1.580	3.870	E
	0.32	16	0.019	0.087	0.127	0.563	0.621	1.859	E
	1.00	0	0.025	0.080	0.701	1.319	1.714	3.304	E
	1.00	5	0.029	0.193	0.450	0.404	0.949	3.727	E
	1.00	16	0.025	0.116	0.077	0.198	0.501	1.710	E
FW Rhizome	0.00	0	0.645	1.217	1.738	2.019	2.091	2.866	L
	0.00	5	0.774	1.400	2.144	2.768	2.973	3.190	L
	0.00	16	0.723	1.513	1.934	1.929	2.438	3.853	L
	0.32	0	0.966	2.009	1.987	2.492	3.026	4.082	L
	0.32	5	0.961	1.559	2.226	2.763	3.480	3.726	L
	0.32	16	0.608	1.353	1.639	2.470	2.640	2.916	L
	1.00	0	0.982	1.455	2.640	2.545	2.227	3.932	L
	1.00	5	0.703	1.949	2.627	2.157	2.855	4.410	L
	1.00	16	0.579	1.138	1.033	1.865	3.141	3.255	L

Table 2.1 (continued)

	Treatment (μM)		Weeks						
Response	BA	MeJa	1	2	3	4	5	6	Fit Type
DW Shoot	0.00	0	0.032	0.032	0.088	0.119	0.162	0.293	E
	0.00	5	0.040	0.040	0.051	0.114	0.195	0.152	E
	0.00	16	0.035	0.035	0.036	0.030	0.089	0.198	E
	0.32	0	0.046	0.046	0.078	0.131	0.151	0.275	E
	0.32	5	0.018	0.018	0.055	0.097	0.146	0.257	E
	0.32	16	0.010	0.010	0.029	0.065	0.078	0.120	E
	1.00	0	0.026	0.026	0.082	0.141	0.130	0.257	E
	1.00	5	0.017	0.017	0.058	0.055	0.101	0.201	E
	1.00	16	0.011	0.020	0.022	0.053	0.074	0.126	E
DW Root	0.00	0	0.008	0.008	0.021	0.064	0.078	0.124	E
	0.00	5	0.010	0.010	0.023	0.062	0.127	0.161	E
	0.00	16	0.011	0.011	0.011	0.018	0.096	0.144	E
	0.32	0	0.016	0.016	0.029	0.068	0.159	0.164	E
	0.32	5	0.010	0.010	0.020	0.056	0.128	0.185	E
	0.32	16	0.010	0.010	0.012	0.040	0.056	0.090	E
	1.00	0	0.006	0.006	0.055	0.080	0.115	0.171	E
	1.00	5	0.018	0.018	0.031	0.026	0.101	0.167	E
	1.00	16	0.011	0.011	0.008	0.015	0.044	0.109	E
DW Rhizome	0.00	0	0.063	0.123	0.173	0.226	0.223	0.300	L
	0.00	5	0.076	0.143	0.219	0.210	0.297	0.362	L
	0.00	16	0.070	0.162	0.191	0.212	0.278	0.412	L
	0.32	0	0.091	0.213	0.190	0.232	0.281	0.403	L
	0.32	5	0.085	0.161	0.227	0.295	0.363	0.344	L
	0.32	16	0.062	0.151	0.161	0.246	0.265	0.309	L
	1.00	0	0.093	0.146	0.297	0.267	0.216	0.390	L
	1.00	5	0.075	0.218	0.274	0.241	0.298	0.428	L
	1.00	16	0.066	0.120	0.105	0.197	0.312	0.334	L

Table 2.1 (continued)

	Treatment (μM)		Weeks						
Response	BA	MeJa	1	2	3	4	5	6	Fit Type
FW Total	0.00	0	0.865	1.721	3.277	4.852	5.160	9.127	E
	0.00	5	0.920	1.974	3.124	5.386	6.788	7.684	E
	0.00	16	0.857	2.068	2.434	2.466	4.553	9.004	E
	0.32	0	1.191	2.823	3.449	5.551	7.746	12.014	E
	0.32	5	1.192	1.913	3.079	4.896	6.911	11.096	E
	0.32	16	0.774	1.577	2.094	3.852	4.116	6.291	E
	1.00	0	1.155	1.859	4.529	5.916	5.836	10.795	E
	1.00	5	0.846	2.344	3.822	3.200	5.123	10.979	E
	1.00	16	0.699	1.469	1.345	2.644	4.564	6.589	E
DW Total	0.00	0	0.103	0.163	0.283	0.408	0.463	0.717	E
	0.00	5	0.126	0.192	0.292	0.385	0.618	0.674	E
	0.00	16	0.116	0.208	0.238	0.260	0.462	0.754	E
	0.32	0	0.152	0.275	0.298	0.431	0.591	0.842	E
	0.32	5	0.113	0.189	0.302	0.448	0.636	0.786	E
	0.32	16	0.082	0.170	0.201	0.351	0.399	0.519	E
	1.00	0	0.125	0.178	0.433	0.488	0.461	0.818	E
	1.00	5	0.109	0.252	0.363	0.322	0.500	0.796	E
	1.00	16	0.087	0.150	0.134	0.265	0.430	0.569	E

Factor	FW Shoot	FW Root	FW Rhizome	DW Shoot	DW Root	DW Rhizome	FW Total	DW Total
MeJa	*	*	*	*	*	*	*	*
BA	-	*	*	*	*	*	*	-
MeJa*BA	*	*	*	*	*	*	*	*

* denotes cell significance at α = 0.01

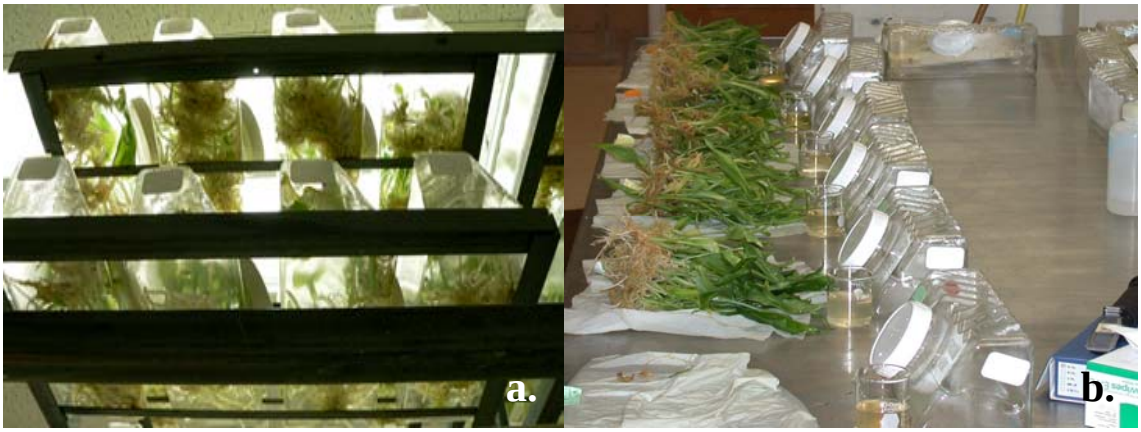


Figure 2.1. Large vessels at time of harvest on rocker-platform (a) and contents of large vessels immediately after harvest (b).

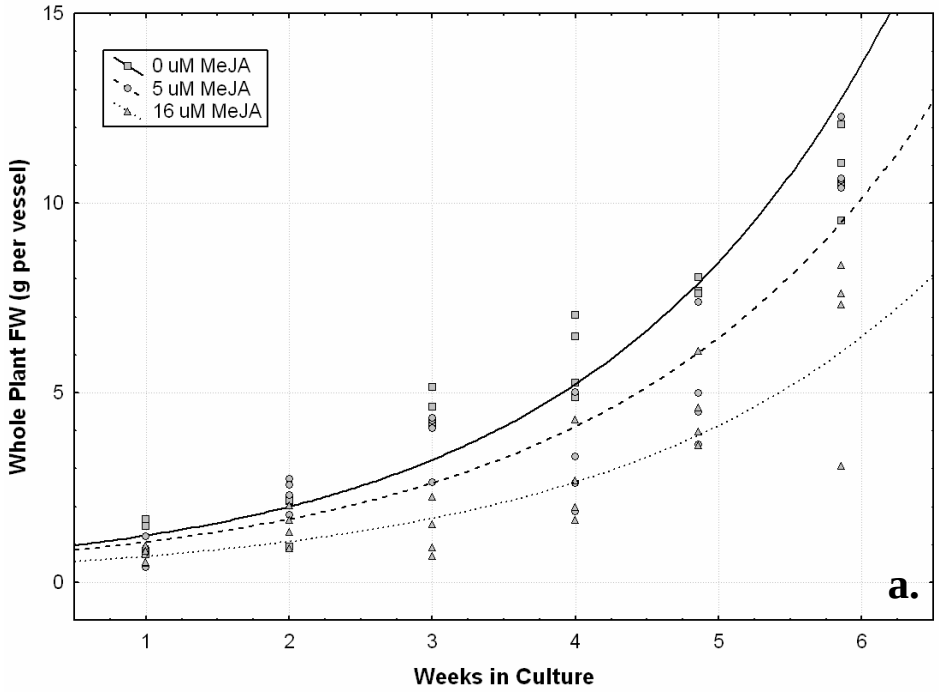
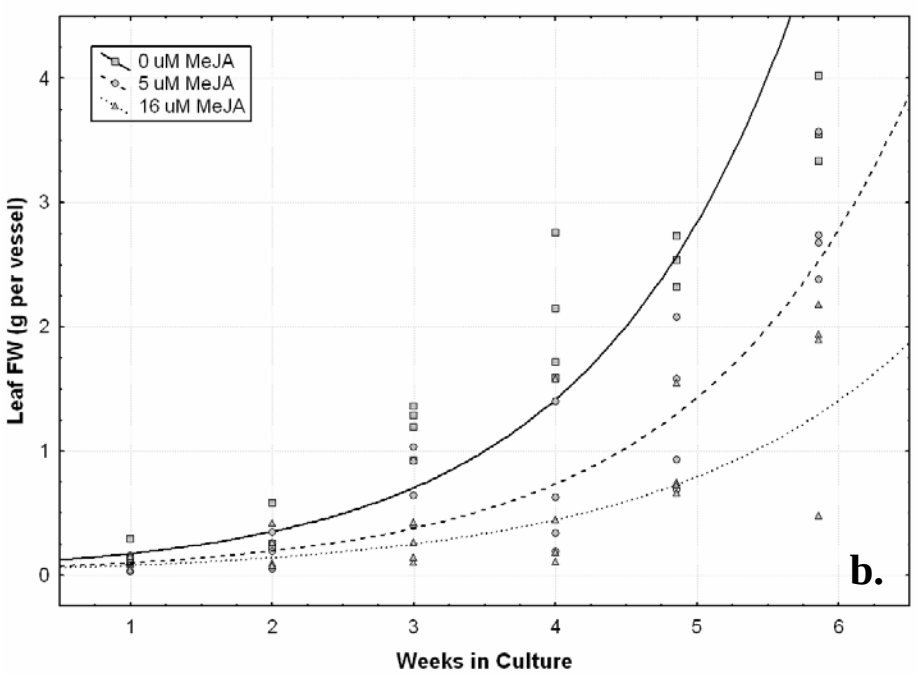
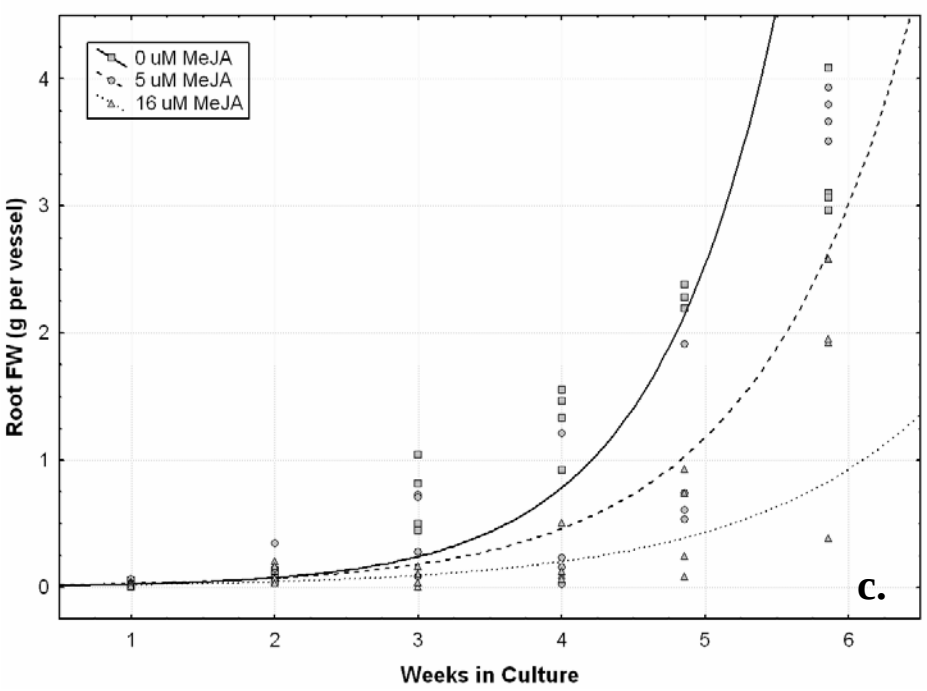


Figure 2.2. Whole plant FW (a), leaf FW (b), and root FW (c) of turmeric plantlets from the short-term time course in small vessels over six weeks with 1 µM BA (n=4).

Figure 2.2 (continued)





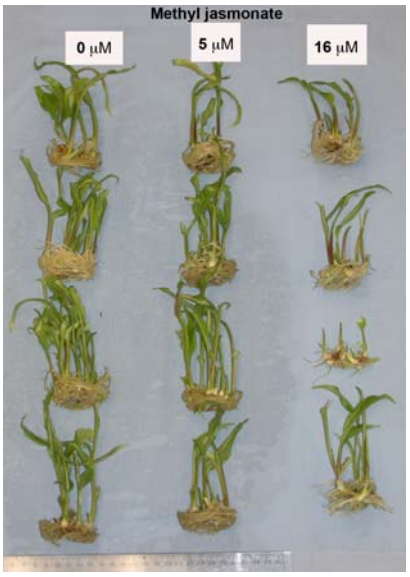


Figure 2.3. Plantlets from the short-term time course that were grown for six weeks in 1 µM BA and 0, 5, and 16 µM MeJa.

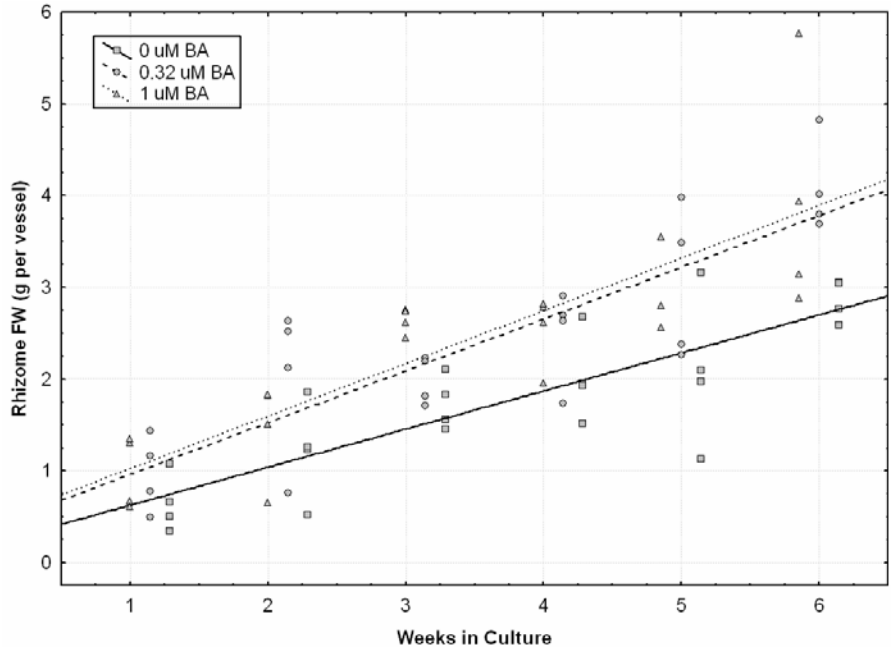


Figure 2.4. Rhizome FW of turmeric plantlets grown for six weeks in 0, 0.32, and 1 µM BA from the short-term time course in small vessels with 0 µM MeJa (n=4).

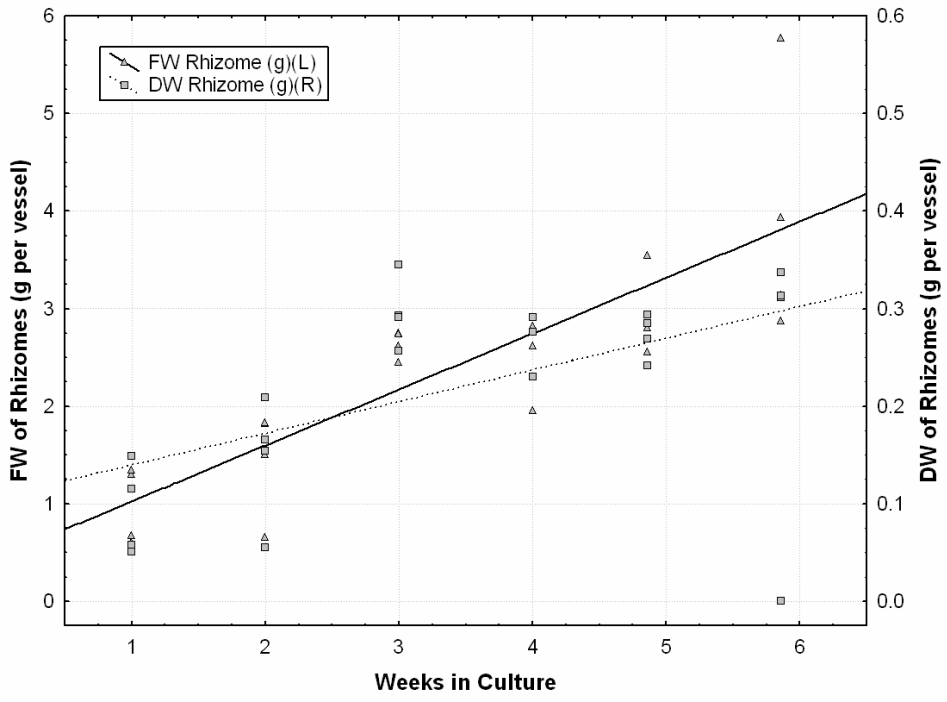


Figure 2.5. FW and DW of turmeric rhizomes grown in the short-term time course in small vessels for six weeks (n=4). Data presented are those for the 1 µM BA and 0 µM MeJa treatment condition. Each line fit was entirely within the 95% confidence interval (not shown) of the other line.

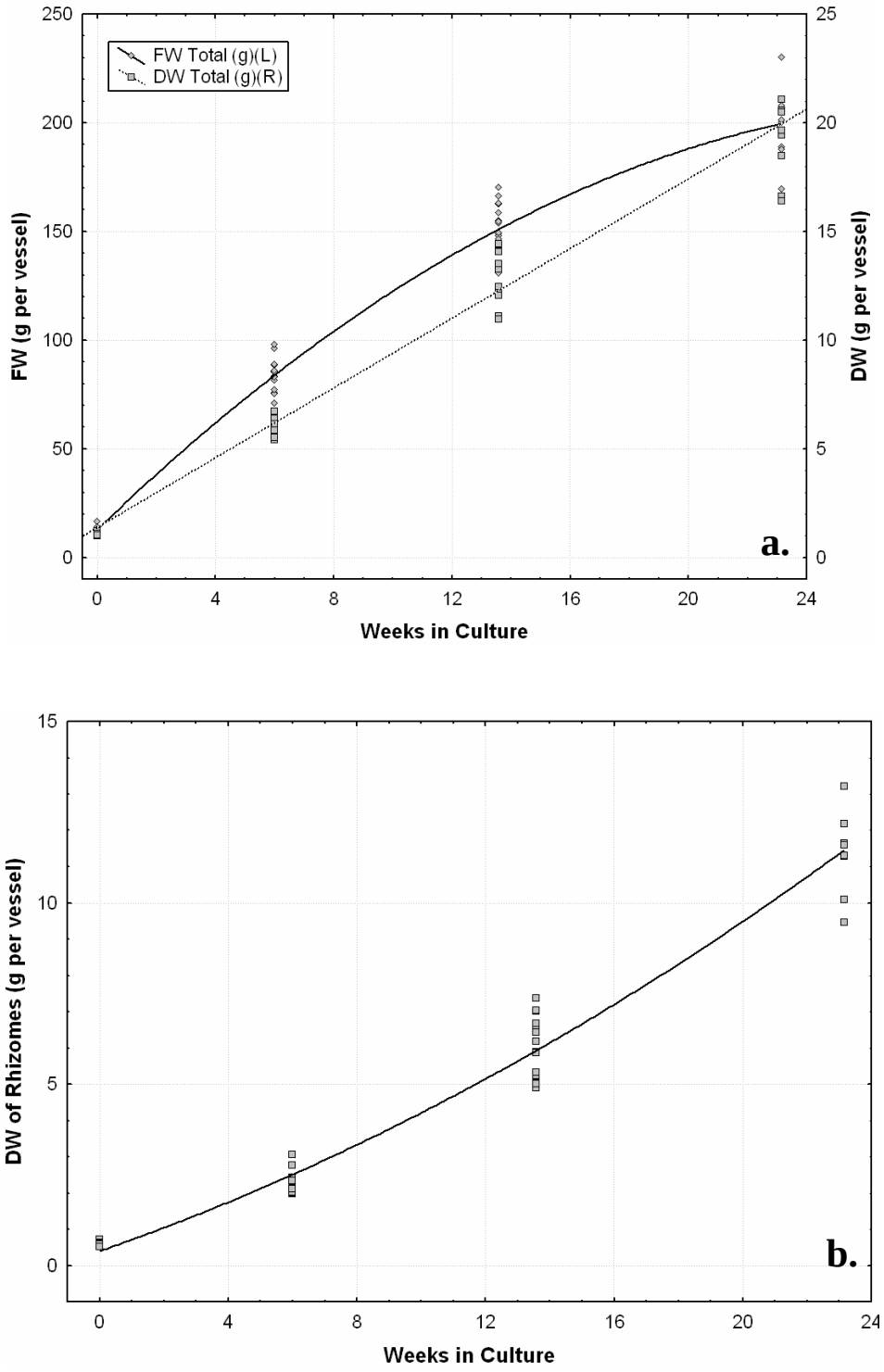
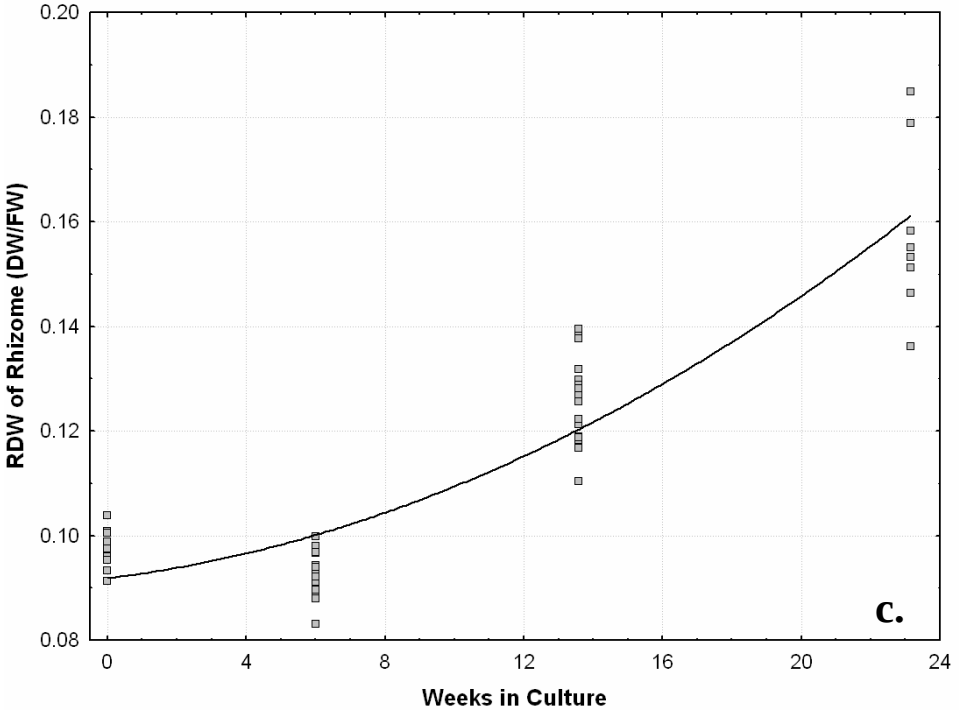


Figure 2.6. Whole plant FW and DW (a), rhizome DW (b), and rhizome RDW (c) of turmeric plantlets from the long-term time course (n=2). Data was pooled for PGRs.

Figure 2.6 (continued)



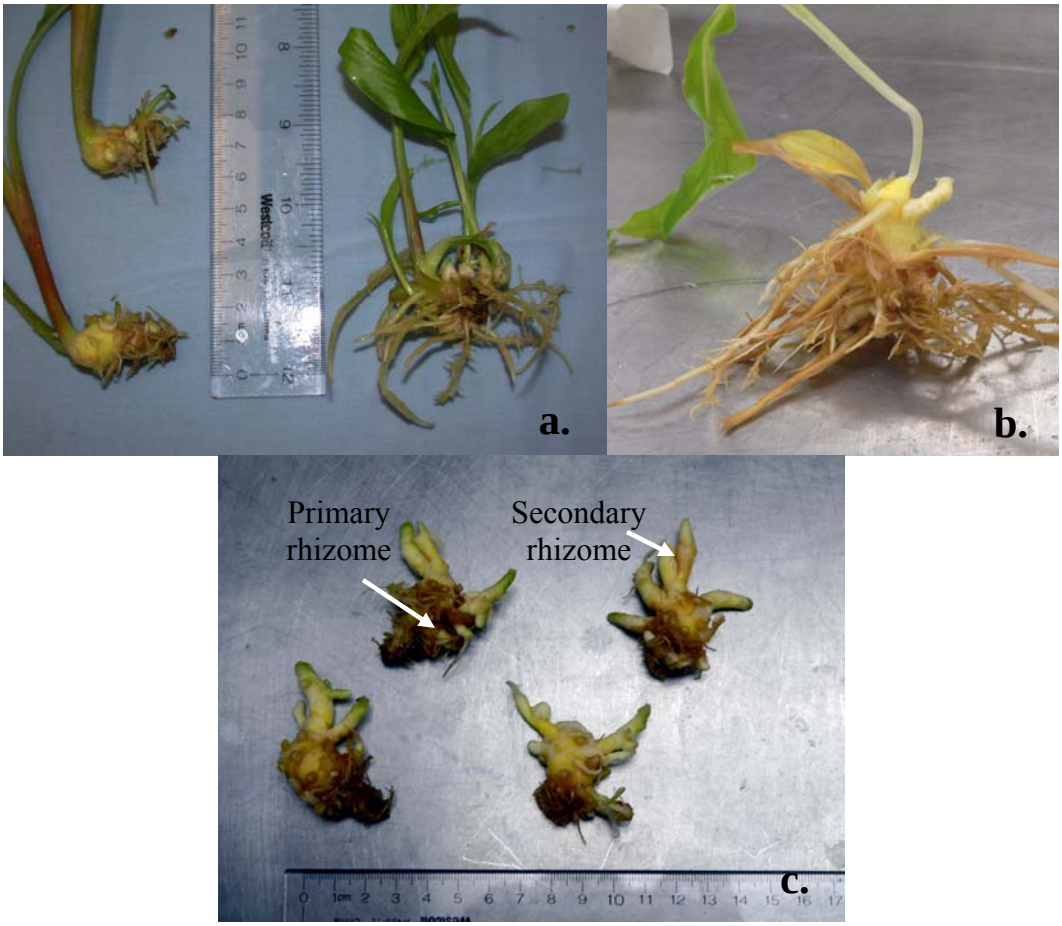


Figure 2.7. Microrhizome development after 14 weeks in the long-term time course (a). Secondary rhizomes were apparent when the surface layers were peeled back, as shown. After 23 weeks, shoots were in atrophy and primary and secondary rhizomes were enlarged (b). Most of the plantlets had several secondary rhizomes (c).

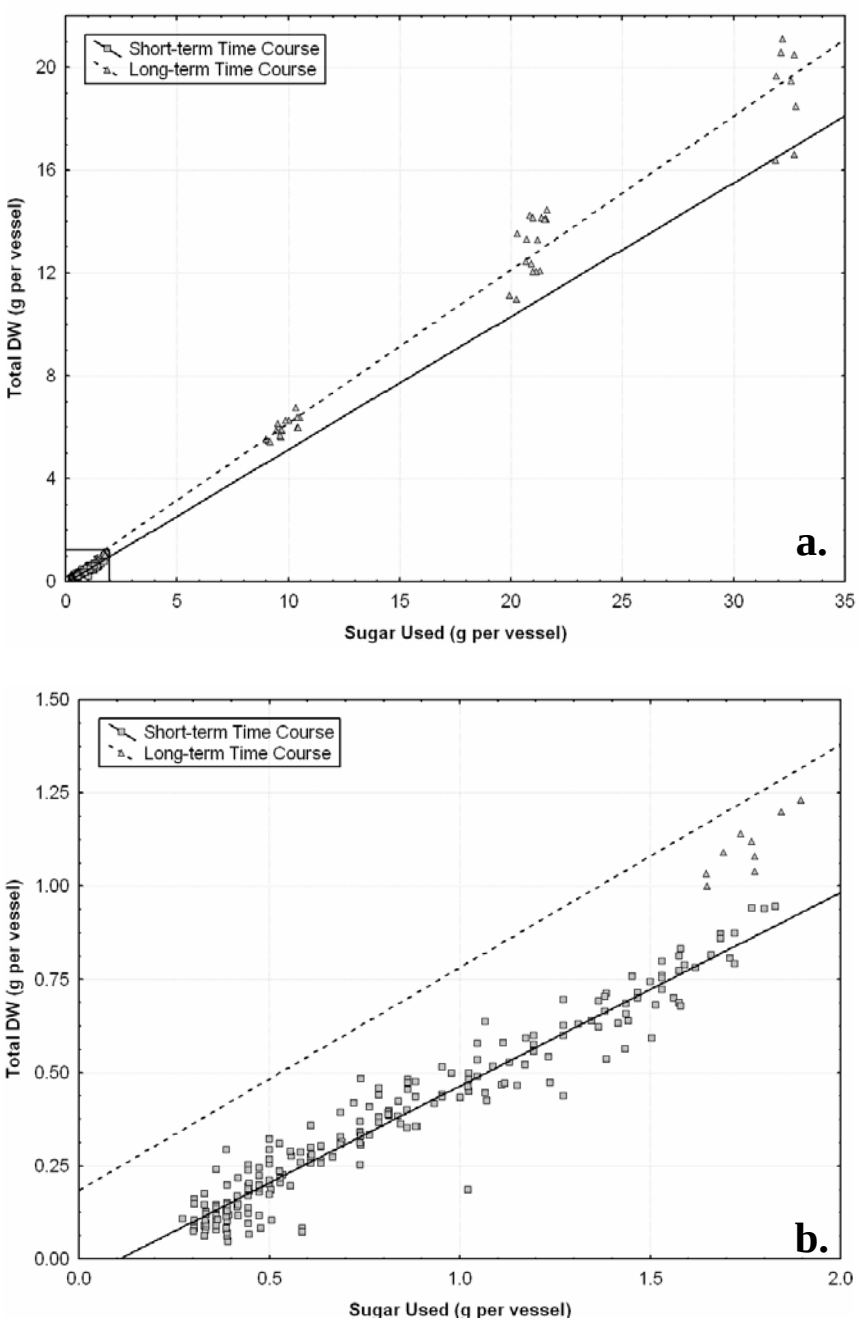


Figure 2.8. The relationship between whole plant DW and sucrose use is shown for the short (n=4) and long-term (n=2) time courses (a). All of the data points from the short-term time course fall within the rectangle at the lower left corner of the scatterplot. This rectangle is enlarged (b) to show adequate resolution for the data from the short-term time course. Sucrose use was nearly directly related to the dry weight in the short-term (Total DW = -0.05 + 0.52 (Sugar Used), $R^2 = 0.94$) and long-term (Total DW = 0.18 + 0.60 (Sugar Used), $R^2 = 0.98$) studies.

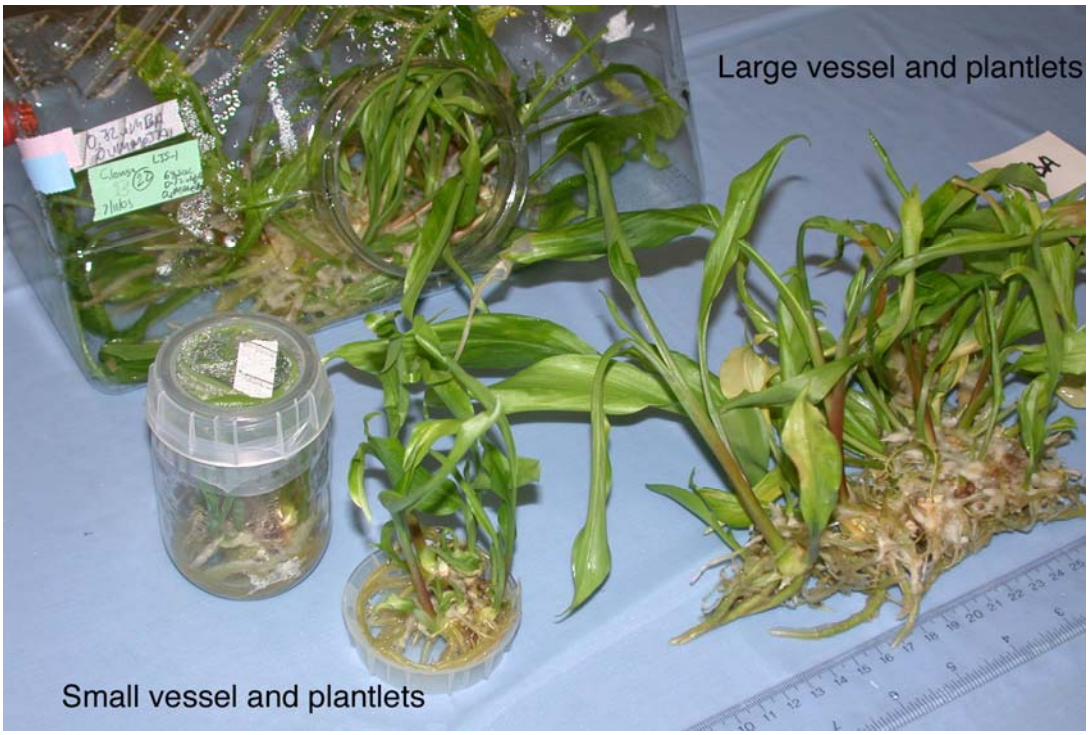


Figure 2.9. Turmeric plantlets that were grown in small jars and large vessels for 6 weeks. Microrhizomes are covered by root and leaf tissue layers and are not readily apparent.

CHAPTER THREE

SECONDARY METABOLISM INDUCING TREATMENTS DURING DEVELOPMENT OF TURMERIC (*CURCUMA LONGA* L.) RHIZOMES IN VITRO

Abstract

Turmeric (*Curcuma longa* L.) plants were grown in vitro for 17 or 22 weeks as a fed-batch culture in 2.5 L vessels. MS liquid medium was restored to 6 % sucrose twice during the experiment. Various methods were employed in attempts to upregulate secondary metabolism. A first experiment exposed four clones to phenylalanine and/or methyl jasmonate (MeJa) from week 12 to 17. In a second experiment on one clone, short-term exposure (1.5 weeks) to proline, a natural proline-rich fish extract, MeJa, and chitosan began during the 20th week of culture. This experiment also included a nitrogen stress treatment (weeks 16-22). The 5 week phenylalanine and MeJa treatments lowered biomass accumulation and antioxidant capacity of the tissue. The magnitude of antioxidant depression was dependent on genotype. Within each genotype, the degree of depression was similar for phenylalanine and MeJa, alone and in combination. In the second experiment, only the low nitrogen treatment yielded an increase in phenolic content compared to untreated microrhizomes. Nitrogen stressed plants also had less leaf growth, but rhizome mass was unaffected. None of the short-term treatments had a significant effect on biomass, antioxidant capacity, or phenolic content. None of the treatments significantly affected radical scavenging although the low nitrogen treatment may have improved this activity ($p = 0.1207$). Results indicated that plants grown in a high nitrogen MS media were not responsive to elicitation.

Abbreviations: Benzyladenine – BA; Dry Weight – DW; Fresh Weight – FW; Gallic Acid – GA; Methyl Jasmonate – MeJa; Relative Dry Weight (DW/FW) – RDW

Introduction

Turmeric (*Curcuma longa* L.) has been used in traditional Indian medicine (Ayurvedic Medicine) for hundreds of years and has recently gained increased attention due to its significant medicinal potential. Commercially, it is used as a dye, spice, and industrial starch. The characteristic yellow-orange curcuminoids found in the rhizomes can be used to make a yellow textile dye. It is also a major ingredient for making curry powder, which is commonly consumed in the countries of southern and eastern Asia and is a major part of most curries.

Much work has been carried out on the antioxidant and related anti-cancer activities of compounds extracted from turmeric rhizomes. The curcuminoids are major antioxidative compounds of turmeric. Many other compounds, including volatile essential oils, also possess antioxidant properties (Duke, 2004). Curcumin may suppress cancer development by helping inhibit enzymes which lead to tumor production (Surh, 2002). Curcumin also prevents cancer along with inflammation by inducing production of enzymes used to detoxify electrophilic species produced in lipid peroxidation (Piper et al., 1998). Verma et al. (1997) showed that curcumin inhibited the growth of human breast cancer cells. Curcumin is also a potent quencher of singlet oxygen species (Das and Das, 2002). Methanolic extracts of turmeric were found to inhibit cyclooxygenase and nitric oxide synthase, enzymes responsible for production of prostaglandins and nitric

oxide; these two species are often implicated in many detrimental processes such as inflammation and carcinogenesis (Hong et al., 2002).

Many attempts have been made to use plant cell culture for production of plant secondary metabolites, but by far, most of these attempts have not been cost effective, and only four commercially viable systems have been created (Alferman et al., 2003). Several factors were blamed for this failure including lack of storage cells for accumulation of secondary metabolites, low yield, and a high cost of equipment. Hairy root culture has better yield using differentiated organs. We have used whole plant culture in a simple mechanical system for production of differentiated microrhizomes with potent accumulation and storage of secondary metabolites (Adelberg and Cousins, 2006; Cousins et al., 2007). A wide variety of biochemical strategies have been utilized to enhance in vitro production of secondary metabolites.

Plant defense hormones such as jasmonates have been shown to induce secondary metabolite production (Gaisser and Heide, 1996; Gundlach et al., 1992; El-Sayed and Verpoorte, 2002; Zabetakis et al., 1999). Jasmonic acid was also found to stimulate storage organ formation in garlic with a 10 μM solution being optimal (Kim et al., 2003). Phenylalanine ammonia lyase, a key enzyme that links primary metabolism to secondary metabolism by serving as a catalyst in the deamination of phenylalanine, was found to be activated by exposure to methyl jasmonate (Sharan et al., 1998). This is considered to be the first step in the shikimic acid pathway.

The precursors in phenolic biosynthesis are the products of the shikimic acid pathway (Bruneton, 1999). Phenylalanine is a precursor for most phenolic compounds in

plants and has been successfully used to induce metabolite production in vitro in many different plant systems (Ibrahim, 1987; Fett-Neto et al., 1994; Petersen, 1999).

Fungal elicitation works as an inducer of secondary metabolism. Exposure of basil to chitosan (a fragment of fungal cell wall polysaccharides) caused significant increases in medicinal compound production (Kim et al., 2005). Fungal mycelium homogenate caused production of antifungal monoterpenes in *Piqueria trinervia* (Saad et al., 2000). For example, chitosan hydrolysates increased paclitaxel production in cell cultures of *Taxus canadensis* (Linden and Phisalaphong, 2000).

Exposure of plant materials to the amino acid L-proline has been shown to increase carbon flow through the pentose phosphate pathway by altering energetic metabolism. This leads to increased activity from the shikimic acid pathway and phenyl propanoid pathways thereby inducing metabolite production in many plant species. Proline and one of its analogs, hydroxyproline, have been shown to cause increases in phenolic and rosmarinic acid contents of thyme (Kwok and Shetty, 1998). Similar results were found for oregano (Yang and Shetty, 1998). Duval and Shetty (2001) showed that phenolics and antioxidant activity were induced by proline.

Lowering the concentrations of inorganic nitrogen has been shown to lead to increased metabolite production. Flavonol accumulation was promoted in plants that were nitrogen deficient (Stewartet al., 2001). Nitrogen deficiency led to increased levels of phenolics and increased activity of phenylalanine ammonia lyase (Kovacik et al., 2007). In tobacco, nitrogen deficiency led to a shift from nicotine production to production of more carbon rich metabolites (Fritz et al., 2006). Lowering nitrogen

concentration from that contained in MS media led to increases in solasodine production from nightshade (Jacob and Malpathak, 2005). The Murashige and Skoog (1962) tissue culture medium (rich in inorganic nitrogen – 20 mM ammonium and 40 mM nitrate) is used most commonly for herbaceous plants (George et al., 2008). This includes all cited studies with turmeric (Babu et al., 2007, and references therein).

As a part of any plan to enhance secondary metabolite production in a plant or organ culture system, two factors must be considered – biomass production and metabolite concentration in tissue. Biomass and microrhizome development were addressed in the previous chapter (Chapter 2).

In our current study, the antioxidant potential and phenolic content of in vitro turmeric microrhizomes grown under different conditions were evaluated. First, the effects of MeJa and the phenolic precursor, phenylalanine, alone and in combination, were measured. The complex turmeric extracts that contain many antioxidant constituents were examined using two assays. The DPPH* free radical scavenging assay was used to determine a "primary" radical scavenging potential, and the ferrous iron chelating assay determined a "secondary" radical scavenging potential that demonstrates the extract's ability to chelate metals in biological systems – preventing reactive oxygen species generation.

A second experiment with one selected clone quantified the effects of MeJa (a defense hormone), chitosan (a fungal elicitor), proline (a pentose phosphate pathway upregulator), Gropro (a complex natural extract rich in proline), and nutrient stress (low nitrogen) on antioxidants and total phenolics. Antioxidants were measured (as above)

with the DPPH* free radical scavenging assay. Total phenolics were measured using the total phenolics assay.

Materials and Methods

Chemicals

Ferrozine, ferrous chloride ($FeCl_2$), tris-HCl, 2,2-diphenyl-1-picrylhydrazyl (DPPH*), Folin-Ciocalteau Reagent, sodium carbonate (Na_2CO_3), and proline were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 2,6-di-tert-butyl-4-methylphenol (BHT) was purchased from ACROS (NJ). Methanol was obtained from Fisher Scientific (Suwanee, GA). Ethylenediaminetetraacetic acid (EDTA) was purchased from JT Baker Chemical Co. (Phillipsberg, NJ). Methyl jasmonate and L-phenylalanine were purchased from PhytoTechnology Laboratories (Shawnee Mission, KS). Gropro and chitosan were obtained from Dr. Kalidas Shetty at the University of Massachusetts – Amherst.

Phenylalanine and MeJa

Four accessions of turmeric, *Curcuma longa* L., (L22-5, L35-1, L43-4, and L50-3) were obtained from the University of Arizona Southwest Center for Natural Products Research and Commercialization. Stage I was prepared by dissecting the quiescent shoot tips from rhizomes, immersing them in full-strength commercial bleach (Clorox™, 5.25% sodium hypochlorite) for 30 seconds, allowing them to air dry on the hood surface, and placing them in PGR free liquid modified MS medium (Murashige and

Skoog, 1962) that included: 170 mg NaH_2PO_4, 100 mg myo-inositol, 0.25 mg nicotinic acid, 0.25 mg pyridoxine hydrochloride, 0.05 mg thiamine hydrochloride, and 30 g sucrose per liter. Medium pH was adjusted to 5.7 before being dispensed. Stage I was conducted with 2 ml of medium in flat-bottomed shell vials (diameter 23 mm). Stage II cultures were maintained for at least 6 months by subculture every six weeks in MS media of the above formulation but with 1 μM BA and 60 g sucrose per liter media. Subculture was conducted in 180 ml glass jars containing 30 ml of liquid tissue culture medium. Material was cultured on an orbital shaker (100 rpm) with 25 to 35 μmol s^{-1} m^{-2} photosynthetically active radiation provided by cool white fluorescent tubes with 16 h d^{-1} photoperiod maintained at 24 $\pm$ 2 °C.

Rhizomes were placed in flasks at a rate of 3-4 buds per jar with 30 ml of media per jar. Following the 5 week period in the jars, the plants were moved to a thin-film rocker system as reported in Adelberg and Cousins (2006). The media used was modified MS media of the same formulation as used in the shake flask culture system. Twelve to eighteen buds were placed in each box along with 200 ml of media. Additional media (100 ml) was added to the vessels twice during the experiment, once at 12 weeks and again at 15 weeks. Two boxes from each clone were given media additions of the following types: same as above, same as above plus 10 μM MeJa, same as above plus 6 mM phenylalanine, and same as above plus 10 μM MeJa and 6 mM phenylalanine. There were two replicates of each type of box in the 4 clones x 2 MeJa levels x 2 phenylalanine levels factorial.

Proline, Gropro, MeJa, and nitrogen deficiency

Plant material was similar to that used in experiment one, but only clone L50-3 was utilized. Rhizomes were placed in boxes at a rate of 14 buds per box with 200 ml of media. An additional 200 ml of media with the same formulation as above was added during week 10. Another media addition was made during week 16. The low nitrogen treatment began during week 16 when nitrogen free MS medium was added to three vessels. Vessels for other treatments were supplemented with MS media as in week 10. During week 21, a process of known addition was used to estimate the media volume in each vessel. Sucrose concentrations of media in vessels were measured on a refractometer (Model # N-10E – ATAGO, U.S.A., Inc., Bellevue, WA). Ten ml of a 20 % sucrose MS media was added to all boxes except those assigned to the low nitrogen treatment, and nitrogen free media with 20 % sucrose concentration was given to the low nitrogen treatment. Using the volume estimates, 2 ml treatment solutions were prepared so as to create specific vessel media concentrations of the remaining treatments. Proline (2 mM), Gropro (natural source of proline – 5 ml l^{-1}), chitosan (200 mg l^{-1}), MeJa (10 μM) were applied through the addition of 2 ml solution in distilled deionized H_2O.

Tissue harvesting and processing

Plants in the first experiment were harvested after 17 weeks while plants in the second experiment were harvested at 22 weeks. Media volume, concentration of sucrose remaining in the media, and number of buds were tabulated for each vessel. Elongated leaves, rhizomes, and roots were separated and massed fresh. Portions of tissue were

dried to calculate DW percentages. Twenty g portions of rhizome tissue were separated from the bulk and frozen separately for use in fresh extractions. All rhizome tissue was stored in the absence of light at -20 ºC prior to extraction.

Extractions, processing, and storage

The 20 g tissue samples were thawed, shredded, and placed in cellulose extraction thimbles. The thimbles were then inserted into a soxhlet apparatus equipped with a condenser. Methanol in the amount of 250 ml was placed in a 500 ml round bottomed flask attached to the base of the soxhlet. The mantle was set to 40 % of capacity. Each extraction was allowed to continue for 12 h. In the first experiment, extracts were allowed to cool to room temperature and gravity filtered through two 20 g portions of sodium sulfate yielding a final extract volume of approximately 200 ml. In the second experiment, the volume of these extracts was standardized to 230 ml prior to a single filtration through a 20 g portion of sodium sulfate. A 50 ml aliquot of each extract was concentrated to 20 ml using a vacuum rotary evaporator. In the case of the first experiment, each of the concentrated extract samples was serially diluted by half until 8 samples of differing concentrations were available for use in the assays to follow. Concentrations ranged from approximately 26 g l^{-1} to 0.2 g l^{-1}. In the second experiment, extract samples were serially diluted by half until 7 samples were available for use in assays. Concentrations ranged from approximately 20 g l^{-1} to 0.2 g l^{-1}. All concentrations were calculated on a rhizome DW basis. Extracts were stored in the absence of light at -5 ºC until assays could be preformed.

Free radical scavenging

Free radical scavenging effect was determined using the free radical generator DPPH* (2,2-diphenyl-1-picrylhydrazyl) by a similar method to that used by Yamaguchi et al. (1998). Two hundred µl aliquots of the serially diluted extract samples were placed in 12 x 75 mm culture tubes with 800 µl of Tris-HCl pH 7.4. One thousand µl of 500 µM DPPH* solution were added to the resulting mixture. The reaction mixture was thoroughly mixed using a vortex and placed in the dark for 20 minutes. After the dark treatment, absorbance was measured via spectrophotometer at 517 nm on a Spectronic 20 Genesys™ spectrophotometer (Fisher Scientific, Fairlawn, NJ). Scavenging activity was calculated via Equation 1.

$$\text{Scavenging Activity (\%)} = \left(1 - \frac{\text{Absorbance of Sample at 517 nm}}{\text{Absorbance of Control at 517 nm}}\right) \times 100$$

(Equation 1)

Fe^{2+} chelating effect

Ferrous iron chelating effects were measured using the method explained by Decker and Welch (1990) with some modification. This assay used the formation of a ferrous iron ferrozine complex to spectrophotometrically monitor the iron chelating ability of the plant extracts observed in this experiment. Eight hundred µl aliquots of the serially diluted extracts were placed in 12 x 75 mm culture tubes with 200 µl of 0.2 mM $FeCl_2$ and 1 mM Ferrozine. The mixtures were then thoroughly shaken and allowed to stand for 10 min at room temperature. The absorbances were then measured at 562 nm on

a Spectronic 20 Genesys™ spectrophotometer (Fisher Scientific, Fairlawn, NJ), and the chelating effects were determined via Equation 2.

$$\text{Chelating Effect (\%)} = \left(1 - \frac{\text{Absorbance of Sample at 562 nm}}{\text{Absorbance of Control at 562 nm}}\right) \times 100$$

(Equation 2)

Total phenolics

The concentration of total phenolic compounds in the extracts was quantified as equivalent amounts of gallic acid (GA). A 200 µl aliquot of rhizome extract was placed in a 16 x 100 mm culture tube followed by 800 µl ddi H_2O, 4 ml saturated Na_2CO_3 solution and 5 ml of 0.2 N Folin-Ciocalteau reagent. This mixture was vortexed and allowed to incubate for 2 hours at room temperature. Solutions were placed on ice before being read on a Spectronic 20 Genesys ™ spectrophotometer (Fisher Scientific, Fairlawn, NJ) at 765 nm. Results from assays were compared with a gallic acid standard curve and were expressed in µg gallic acid per mg rhizome DW.

Experimental design and analysis

In experiment one, biomass comparisons were made using ANOVA α=0.05. Antioxidant activities of rhizome tissue from the four clones in four treatment conditions were compared in a factorial design. The untreated clones served as controls and were compared with each treatment within clone. DPPH* free radical scavenging and iron chelating curves were established by fitting the data to a hyperbolic tangent function

(Cousins et al., 2007). Estimates of the EC_{50}s (extract concentration that gives 50% of maximum effect) were obtained using the maximum likelihood method, and Wald's *z*-test was used for all comparisons (Casella and Berger, 2001). In experiment two, biomass comparisons were conducted as above. EC_{50} values were calculated and compared using ANOVA α=0.05, and a Dunnett's test was used to compare treatments with the control.

Results and Discussion

At harvest, the large amounts of tissue filled nearly all available space in the vessels (Figure 3.1). Rhizomes were relatively large and contained the bright yellow coloring characteristic of curcuminoids (Figure 3.2). All tissue parts exuded a pleasant odor indicating the presence of volatile compounds.

Phenylalanine and MeJa

MeJa and phenylalanine, alone and in combination, decreased biomass accumulation of turmeric plants in vitro (Table 3.1). The effects of these treatments were not entirely uniform across the four clones.

Total FW was impacted by phenylalanine, MeJa, and clone. Phenylalanine and MeJa had large negative impacts on whole plant FW production by the plantlets. L22-5 produced the least FW followed by L35-1 with L43-4 and L50-3 producing the most FW. Microrhizome FW and RDW (ratio of DW/FW) were not affected by MeJa or phenylalanine. This response variable was impacted only by clone.

In all four clones, phenylalanine and MeJa alone and in combination decreased the antioxidant potential of the rhizome extracts (Table 3.2). Both MeJa and phenylalanine reduced antioxidant potentials by a similar amount, and their combined effect was similar to the effect of either chemical alone. This was consistent for all 4 clones even though there were differences in the size of the response from clone to clone. For example, in the clone with the strongest antioxidant potential (L 22-5), MeJa and phenylalanine increased EC_{50}s by factors of 7.8 and 7.4, respectively. In combination, the two treatments caused the EC_{50}s to increase by a factor of 7. In the clone with the weakest antioxidant potential (L 35-1), phenylalanine or MeJa increased EC_{50}s by a factor of 2.7. In combination, the EC_{50} was increased by a factor of 2.5.

Iron chelation data was inconsistent across clones for the treatments (Table B-1). In hindsight, some EDTA from the medium might have been incorporated into the rhizome where it was extracted and caused unpredictable effects on the chelation assay. Despite this realization, nothing in the chelation assay data caused us to doubt the general conclusion that MeJa and phenylalanine did not enhance the antioxidant potential of the turmeric microrhizomes.

Phenylalanine could have caused feedback inhibition in the shikimic acid pathway, and the MeJa treatment could have had a similar effect. There is also a chance that we induced metabolites that were not detected by our assays and that this enhancement channeled carbon away from the metabolites we measured. Induction may have occurred in the short-term. Effects could have been overshadowed by primary

metabolism depression from the long period of exposure to the compounds. A shorter period of induction was attempted in experiment two.

Proline, Gropro, MeJa, and nitrogen deficiency

We selected the clone L 50-3 from the first experiment as its responses were typical with regard to MeJa and phenylalanine. Treatments caused significant reductions in antioxidant potential. The short-term induction in this second experiment did not alter rhizome biomass, and all the treatments except for the low nitrogen treatment had the same mass as the control. The low nitrogen treatment lowered shoot FW and DW accumulation (Figure 3.3). The ten day treatment period probably did not allow differences in plant growth to become apparent.

The nitrogen deficient plants produced more phenolics than the control (Table 3.3). No other treatment had a significant effect on phenolic production. There was also a difference between the effect of proline and the effect of Gropro in this experiment. The complex natural product, Gropro, produced a significantly larger amount of phenolics than proline.

None of the treatments altered the ability of extracts to scavenge the DPPH* radical to a significant degree (Table 3.3). EC_{50}s of extracts from tissue treated with chitosan, Gropro, nitrogen deficient media, MeJa, and proline were not significantly different from the control. Low nitrogen may have had the effect of decreasing the EC_{50} or increasing scavenging efficacy (low nitrogen vs. control; $p = 0.1207$). Three

replications were utilized due to economy of working in 2.5L vessels over long time frames. Larger numbers of replications may have yielded statistical significance.

Conclusion

In whole plant and organ culture, both concentration of active compounds and biomass must be considered for metabolite production. Turmeric is grown in nitrogen-rich MS medium to promote maximum biomass production. Our attempts at induction of secondary metabolism in the first experiment may have been less effective in this nutrient-rich environment. As indicated by the second experiment, it may not be possible to successfully upregulate secondary metabolite production in turmeric in an environment containing luxuriant amounts of nitrogen. Sugar use has been related to rhizome biomass through primary metabolism (Chapter 2). Optimization of carbon use in a low nitrogen environment may need to precede attempts to upregulate secondary metabolism growth regulators or precursors. This methodology is a powerful tool for eventual better understanding of the metabolite production process and a gateway to construction of a model from sugar use and nutrient status to metabolite production.

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Table 3.1.
Biomass measurements (g) for main responses separated by treatment and clone.

	Treatments		Clone			
Response	Phenylalanine	MeJa	L22-5	L35-1	L43-4	L50-3
FW Shoot	no	no	42.000	44.840	75.345	56.000
	yes	no	49.700	46.520	70.610	72.890
	no	yes	53.900	52.925	68.255	68.835
	yes	yes	46.090	57.750	65.845	68.110
FW Root	no	no	56.500	57.875	79.485	65.000
	yes	no	30.765	40.955	47.930	44.815
	no	yes	36.945	41.620	47.000	50.085
	yes	yes	32.355	41.790	46.600	40.385
FW Rhizome	no	no	42.000	53.865	44.540	50.500
	yes	no	34.790	35.610	38.270	39.420
	no	yes	36.950	37.750	42.030	36.730
	yes	yes	34.305	40.700	42.170	42.110
DW Shoot	no	no	3.695	4.295	5.465	4.370
	yes	no	3.240	4.360	4.155	4.555
	no	yes	3.695	3.930	4.340	4.615
	yes	yes	3.255	4.195	4.080	4.560
DW Root	no	no	2.480	3.055	4.130	3.510
	yes	no	2.235	3.035	3.375	3.100
	no	yes	2.775	3.015	3.365	3.375
	yes	yes	2.540	3.055	3.250	3.140
DW Rhizome	no	no	4.380	5.920	4.410	4.850
	yes	no	4.148	4.071	3.275	3.636
	no	yes	4.234	3.817	3.560	3.400
	yes	yes	3.548	4.171	4.050	3.861

Table 3.1 (continued)

	Treatments		Clone			
Response	Phenylalanine	MeJa	L22-5	L35-1	L43-4	L50-3
FW Total	no	no	140.500	156.580	199.370	171.500
	yes	no	115.255	123.085	156.810	157.125
	no	yes	127.795	132.295	157.285	155.650
	yes	yes	112.750	140.240	154.615	150.605
DW Total	no	no	10.555	13.270	14.005	12.730
	yes	no	9.623	11.466	10.805	11.291
	no	yes	10.704	10.762	11.265	11.390
	yes	yes	9.343	11.421	11.380	11.561
%DW Rhizome	no	no	0.103	0.110	0.099	0.096
	yes	no	0.119	0.114	0.086	0.093
	no	yes	0.114	0.101	0.085	0.093
	yes	yes	0.104	0.103	0.096	0.092

Factor	FW Shoot	FW Root	FW Rhizome	DW Shoot	DW Root	DW Rhizome	FW Total	DW Total	%DW Rhizome
Clone	*	*	-	*	*	-	*	*	*
Phenylalanine	-	*	*	-	*	*	*	*	-
MeJa	-	*	*	-	-	*	*	*	-
Clone*Phenylalanine	-	-	-	-	-	-	-	-	-
Clone*MeJa	-	-	-	-	-	-	-	-	-
Phenylalanine * MeJa	-	*	*	-	-	*	*	*	-
Clone*Phenylalanine*MeJA	-	-	-	-	-	-	-	-	-

* denotes cell significance at $\alpha = 0.05$

Table 3.2.
DPPH* free radical scavenging of methanolic rhizome extracts from four in vitro grown clones of turmeric cultured in normal media compared to turmeric treated with hormone and/or precursor.

Clone	MeJa	Phenylalanine	EC_{50} (gl^{-1})	Variance	Control-Treated	
L22-5	no	no	1.046	0.00816		
L22-5	yes	no	8.190	0.02848	-7.143	***
L22-5	no	yes	7.763	0.18488	-6.717	***
L22-5	yes	yes	7.357	0.13597	-6.311	***
L35-1	no	no	1.816	0.02122		
L35-1	yes	no	4.946	0.04543	-3.131	***
L35-1	no	yes	4.883	0.03317	-3.068	***
L35-1	yes	yes	4.667	0.00216	-2.851	***
L43-4	no	no	1.551	0.00110		
L43-4	yes	no	3.995	0.00759	-2.444	***
L43-4	no	yes	5.162	0.02815	-3.611	***
L43-4	yes	yes	4.289	0.02044	-2.738	***
L50-3	no	no	1.151	0.00239		
L50-3	yes	no	5.057	0.03226	-3.906	***
L50-3	no	yes	4.980	0.04107	-3.829	***
L50-3	yes	yes	5.140	0.06707	-3.989	***

*, **, and *** indicate differences significant at 0.10, 0.05, and 0.01 respectively as determined by Wald's *z*-test.

Table 3.3.
DPPH* free radical scavenging and total phenolics for in vitro grown turmeric clone L50-3 grown in normal media compared to turmeric treated with chemicals designed to induce secondary metabolite production.

Treatment	DPPH* EC_{50} (gl^{-1})	Variance	Control-Treatment		Total Phenolics (µg GA/mg DW)	Variance	Control-Treatment	
Control	4.075	0.09533			41.008	8.86814		
Chitosan	4.588	0.31665	-0.512	-	40.111	6.76497	0.898	-
Gropro	4.070	0.31884	0.006	-	44.005	8.68599	-2.997	-
Low Nitrogen	3.399	0.01396	0.676	†	46.888	3.05680	-5.880	***
Methyl Jasmonate	3.860	0.18952	0.216	-	43.170	0.53329	-2.162	-
Proline	4.364	0.54041	-0.289	-	38.898	3.21886	2.110	-

*, **, and *** indicate differences significant at 0.10, 0.05, and 0.01 respectively as determined by Dunnett's test.
† indicates difference that would be significant at 0.15 (p value = 0.1207).

Figure 3.1. Tissue as viewed through the back of the vessel on the day of harvest (a), and tissue removed from a single box that has been separated by plant organ (leaf, root, and rhizome) (b).

Figure 3.2. Bisected turmeric rhizome shows yellow coloring of curcuminoids (a). Large intact rhizomes displaying yellow coloring and club shaped morphology (b).

Figure 3.3. Comparison of nitrogen stressed plants (right) with plants from a representative control vessel (left).

APPENDICES

Appendix A

Biomass Scatterplots

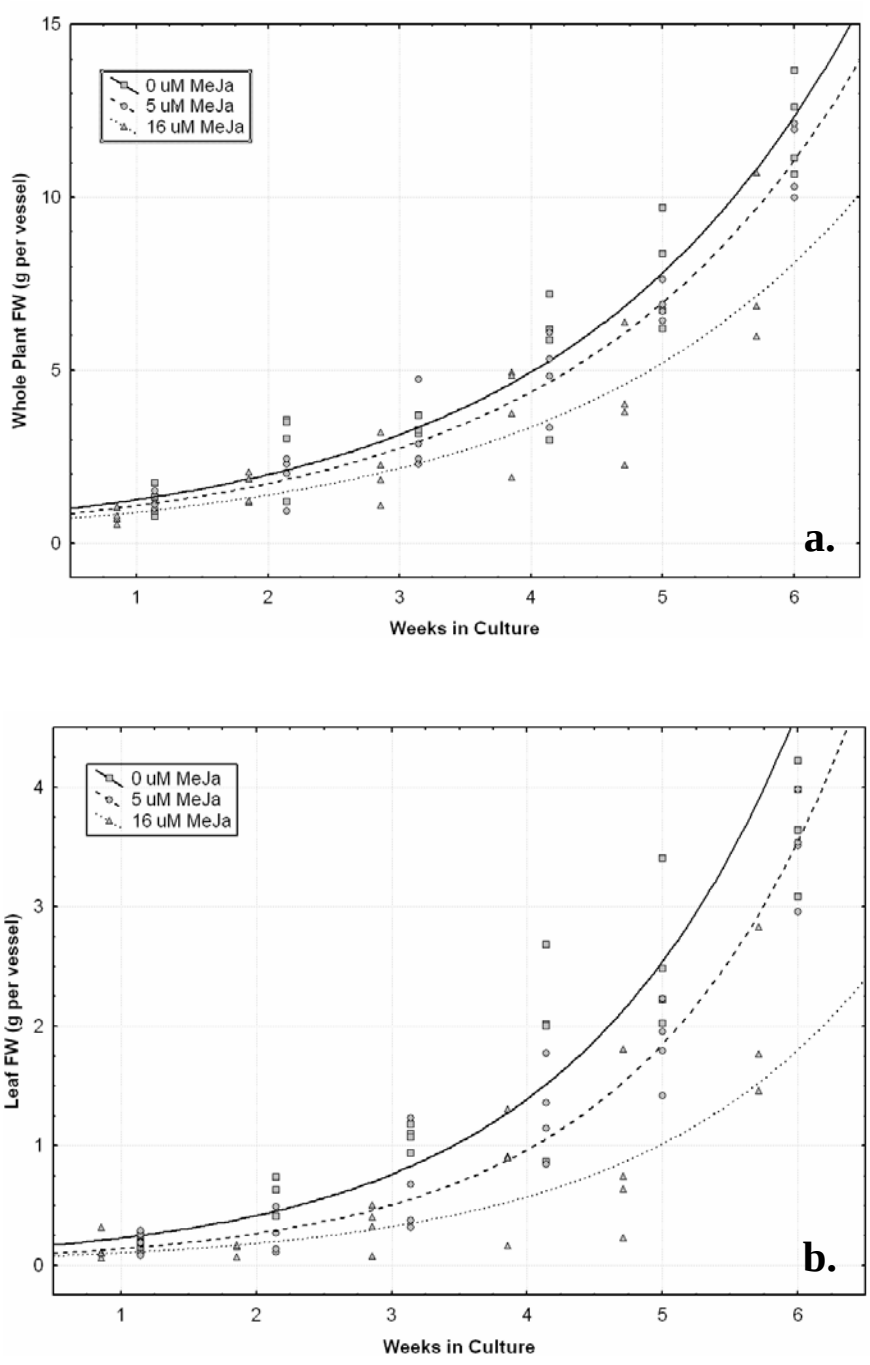
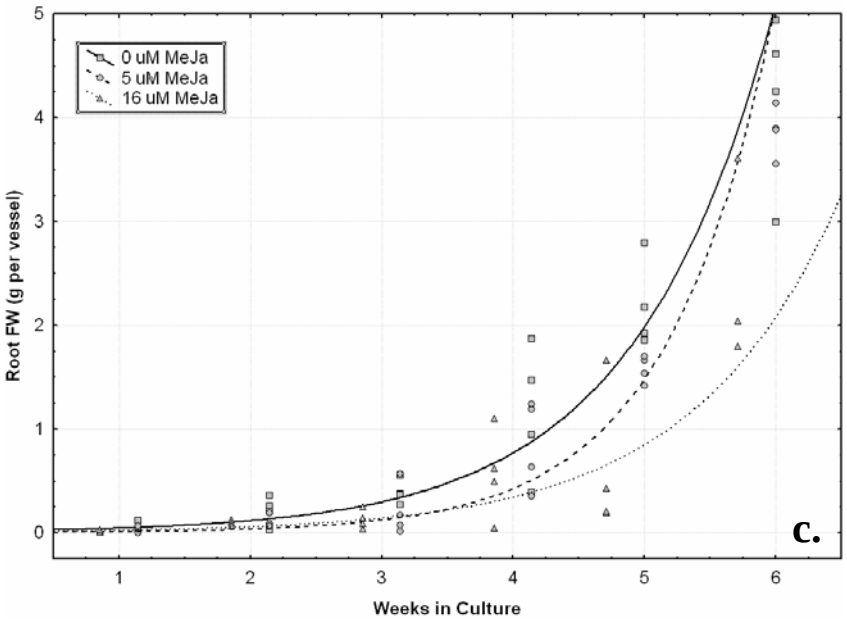


Figure A-1: Whole plant FW (a), leaf FW (b), and root FW (c) of turmeric plantlets from the short-term time course in small vessels over six weeks with 0.32 µM BA (n=4).

(Figure A-1 continued)



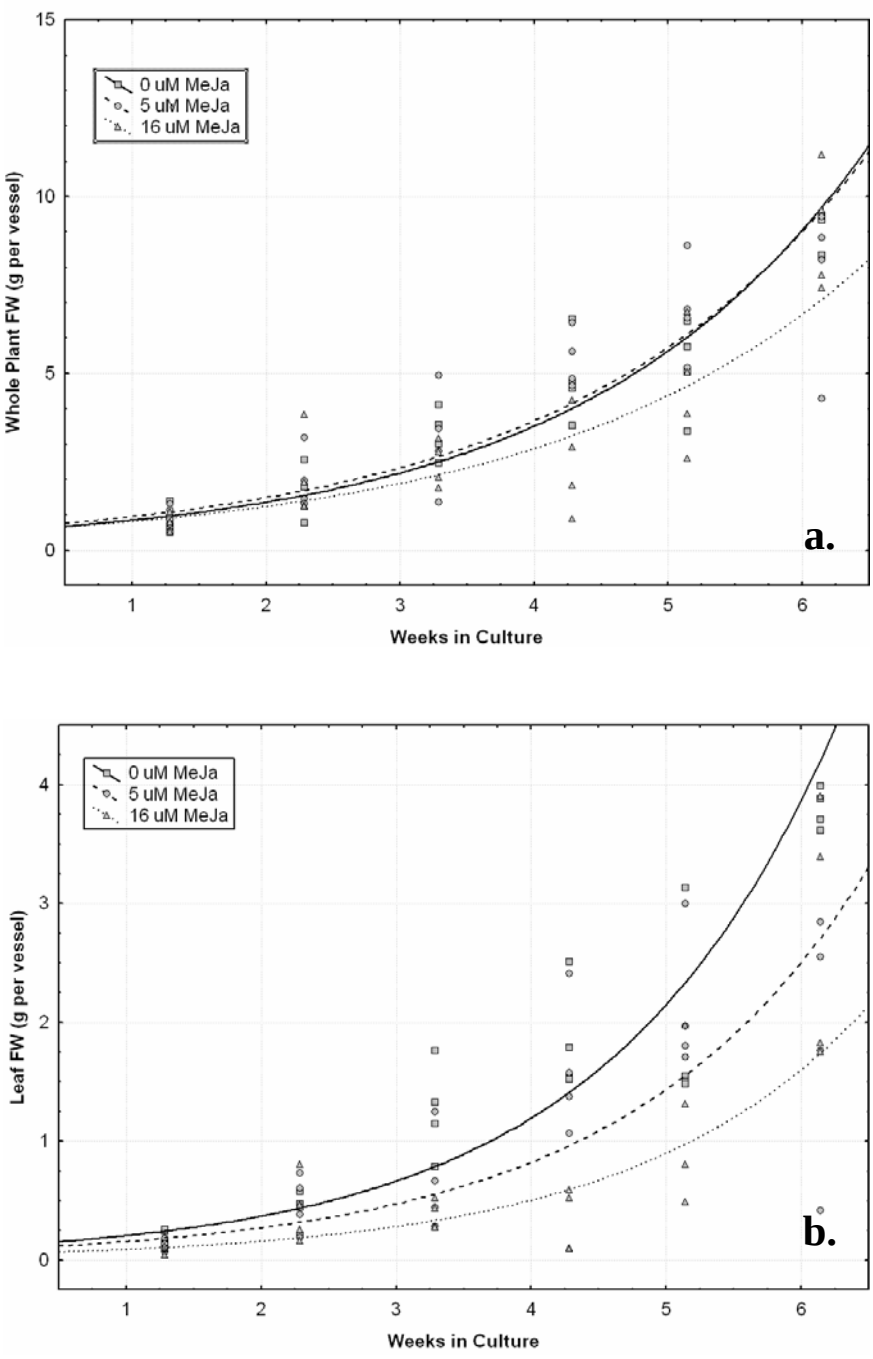
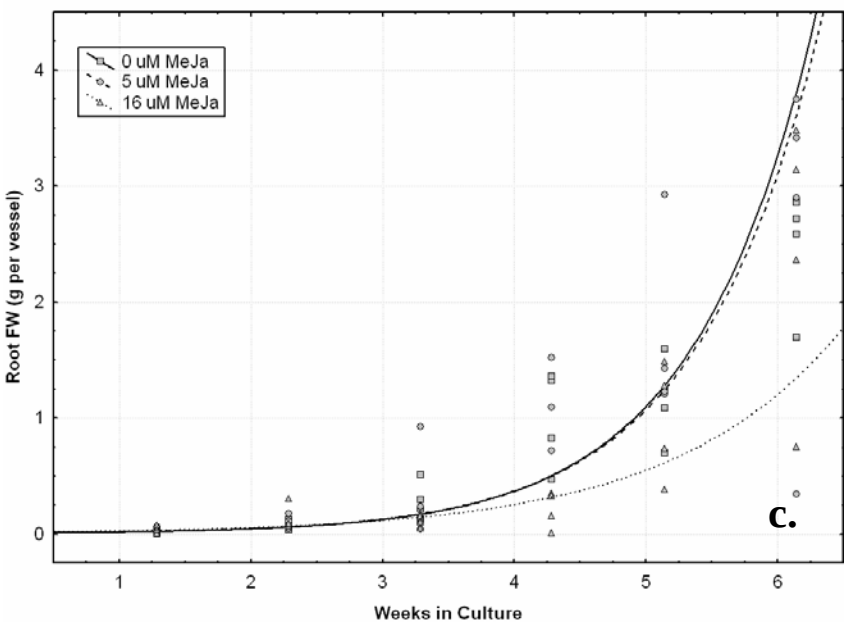


Figure A-2: Whole plant FW (a), leaf FW (b), and root FW (c) of turmeric plantlets from the short-term time course in small vessels over six weeks with 0 μM BA (n=4).

(Figure A-2 continued)



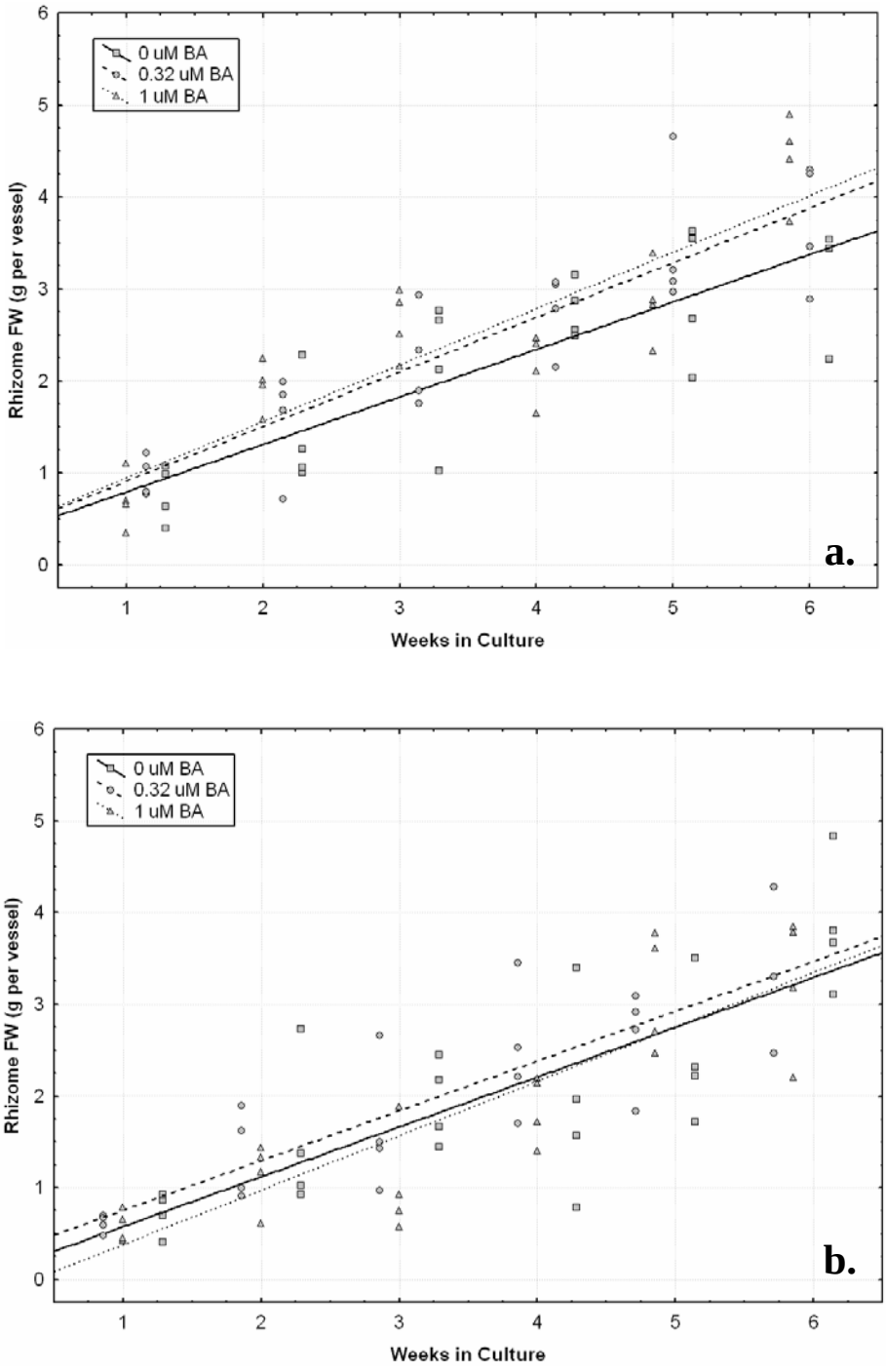


Figure A-3: Rhizome FW of turmeric plantlets from the short-term time course in small vessels over six weeks for BA levels within 5 µM MeJa (a) and 16 µM MeJa (b).

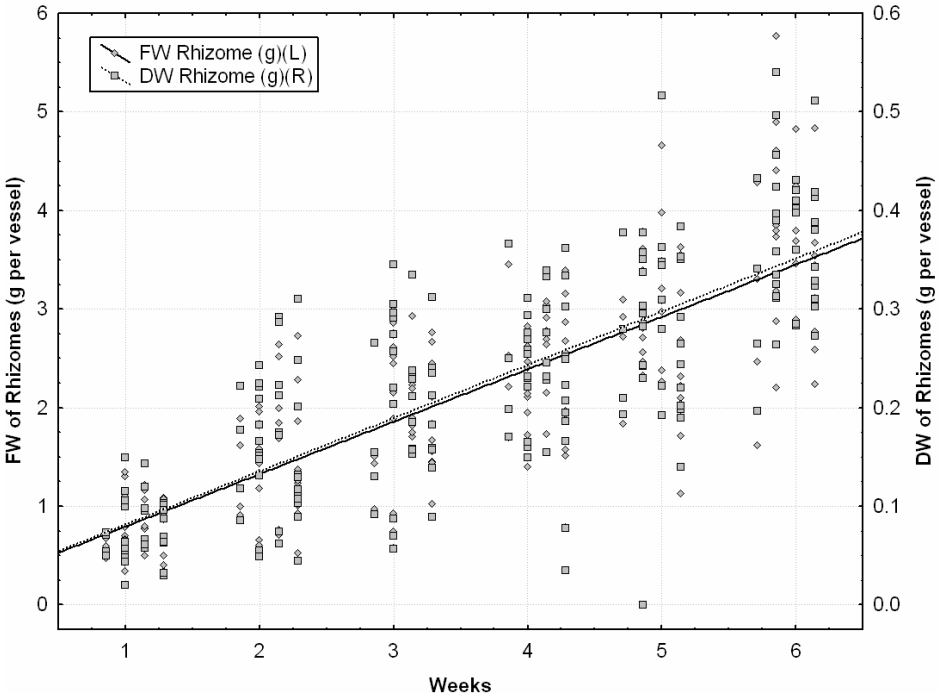


Figure A-4: FW and DW of turmeric rhizomes grown in the short-term time course in small vessels for six weeks (n=4). The line fits for each data set are close enough that they almost appear as a single line.

Appendix B

Iron Chelating Assay Data

Table B-1.
Iron chelation of methanolic rhizome extracts from four in vitro grown clones of turmeric cultured in normal media compared to turmeric treated with hormone and/or precursor.

Clone	MeJa	Phenylalanine	EC_{50} (gl^{-1})	Variance	Control-Treated	
L22-5	no	no	0.931	0.00120		
L22-5	yes	no	0.949	0.00413	-0.018	-
L22-5	no	yes	0.811	0.00073	0.120	***
L22-5	yes	yes	0.899	0.00060	0.032	-
L35-1	no	no	0.961	0.00236		
L35-1	yes	no	0.815	0.00408	0.147	*
L35-1	no	yes	0.886	0.00127	0.075	-
L35-1	yes	yes	1.321	0.00895	-0.360	***
L43-4	no	no	1.462	0.00494		
L43-4	yes	no	1.054	0.00447	0.407	***
L43-4	no	yes	1.909	0.05369	-0.447	*
L43-4	yes	yes	1.248	0.00265	0.214	***
L50-3	no	no	1.227	0.00635		
L50-3	yes	no	1.545	0.01749	-0.318	**
L50-3	no	yes	1.542	0.00551	-0.315	***
L50-3	yes	yes	1.678	0.06296	-0.451	*

*, ** and, *** indicate differences significant at 0.10, 0.05, and 0.01 respectively as determined by Wald's *z*-test.